SEPARATION OF THE MITOGENIC AND SYNERGISTIC ACTIVITIES OF INTERLEUKIN-3

By

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A thesis submitted for the degree of Doctor of Philosophy of the Australian National University.

January 1989

STATEMENT

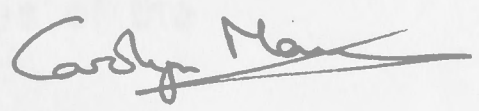
All of the experimental work reported in this thesis was performed by the author alone unless otherwise stated below. The contributions of the people who collaborated in this work were:

Dr A. Hapel and Dr N. Nicola performed the IL-3 binding experiments described in Chapter 3.

Dr M. Kobayashi and Dr J. Salisbury assisted with colony assays.

Mrs L. Preston assisted with labelling cDNA probes used for Northern analysis.

Mr P. Townsend assisted with the separation of AKR spleen cell conditioned medium (Chapter 3).



C. F. L. Morris

January 1989

ACKNOWLEDGEMENTS

I am grateful to the many people who have provided assistance during my years as a student at the ANU. These include my supervisors, Dr Andrew Hapel and Dr Ian Young, who provided guidance during this project; Dr Hugh Campbell, Dr Donna Cohen, and Janet Lee to whom I owe much of my understanding of molecular biology; and Dr David Hume, Dr Sanie Ymer, Garry Mayo, Dr Mark Baker, Dr Rhod Ceredig, Dr Anthony Jaworowski and Dr Robert Cutler who provided helpful advice or materials at various stages of this project. I also acknowledge Dr Masa Kobayashi and Dr Janet Salisbury for assisting with the colony assays.

All of Andrews "team", past and present, have given me continued practical and moral support. Among these I should like particularly to thank Lynette Preston who provided excellent technical assistance and Sue Elsbury who abandoned her assays to help with the preparation of manuscripts and presentations.

Thanks also to Andrew Hapel and Francis Cunningham for their generous hospitality when I first arrived in Canberra; Rosemary Enge for her professional preparation of part of this manuscript; and Evan Ingley for unravelling some of the mysteries of the Macintosh.

Finally, and most of all, thankyou to my family for their love and encouragement "long-distance" and to Brett Allen who has been my psychiatrist, feeder and closest friend during a difficult time.

ABSTRACT

Previous work has shown that IL-3 is a haemopoietic growth factor which stimulates the proliferation of all haemopoetic lineages. However, the data presented here show that there is a considerable strain-dependent variation in the response of murine bone marrow cells to IL-3. Bone marrow cells from most strains including DBA/2 responded well to IL-3 both in colony assays and liquid proliferation assays. In contrast, IL-3 did not support colony formation from A/J or AKR bone marrow cells in soft agar culture and supported only a low level of proliferation in liquid cultures of these cells. Further experiments revealed that the low level of proliferation in liquid assays was cell density dependent and was most likely due to endogenous growth factor(s) induced by IL-3 rather than to any direct mitogenic activity of IL-3.

The combination of M-CSF and IL-3 supported the production of distinctively large colonies from murine bone marrow cells regardless of the strain of the donor mouse. In cultures of A/J bone marrow cells, the synergistic effect of IL-3 and M-CSF was also reflected in an increase in the number and variety of colonies produced. A/J bone marrow cells precultured with IL-3 were not activated to produce large colonies when subsequently cultured with M-CSF alone although the presence of IL-3 in these precultures did maintain M-CSF-reponsive cells which died or lost responsiveness in the absence of exogenous growth factors. Pre-exposure to M-CSF did not overcome the defect in the proliferative response of A/J bone marrow cells to IL-3. Therefore, the direct

mitogenic activity of IL-3 can be separated from its synergistic activity with M-CSF.

Northern analysis revealed that culture with IL-3 enhanced the levels of c-fms (M-CSF receptor) RNA transcripts expressed by A/J bone marrow cells. Thus, IL-3 may synergise with M-CSF primarily by inducing the expression of M-CSF receptors. IL-3 also modulated growth factor expression in haemopoietic cells. Culture with IL-3 elevated the expression of M-CSF RNA transcripts in both A/J and DBA/2 bone marrow cells. No G-CSF, GM-CSF or IL-4 RNA transcripts were found in these cells before or after culture with IL-3. Therefore, endogenous M-CSF induced by IL-3 probably accounts for the cell density dependent proliferative response to IL-3 detected in liquid cultures of A/J bone marrow cells.

IL-3 and Epo synergised to enhance erythroid colony formation in semi-solid cultures of DBA/2 bone marrow cells. Similarly, IL-4 enhanced IL-3-dependent colony formation in such cultures. However, IL-3 combined with Epo or with IL-4 failed to support colony formation from A/J bone marrow cells. Therefore, the interactions of IL-3 with Epo and IL-4 may be dependent on the mitogenic activity of IL-3.

Since IL-3 does not induce the proliferation of bone marrow cells from some strains, the direct mitogenic activity of IL-3 may not be essential for maintaining haemopoiesis. Other actions of IL-3 including the maintenance of factor responsive cells, induction of growth factors and synergism with M-CSF may nevertheless play an important role in blood cell growth and development.

Abbreviations commonly used in this thesis

BFU -E	Burst forming unit -erythroid
-Mk	-megakaryocyte
Bp	Base pair
BPA	Burst-promoting-activity
CFC	Colony forming cell
CFU	Colony forming unit
CFU-S	Spleen colony forming unit
CSF	Colony stimulating factor
DEAE	Diethylaminoethyl
Epo	Erythropoietin
5-FU	5-fluorouracil
G	Granulocyte
GEMM	Granulocyte, erythrocyte, monocyte, megakaryocte
GM	Granulocyte/macrophage
HPP-CFC	High-proliferative-potential colony-forming-cell
Kb	Kilobase
IL	Interleukin
PHSC	Pluripotent haemopoietic stem cell
r	Recombinant
20α-SDH	20α-hydroxysteroid dehydrogenase

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Chapter 1

Literature Review

INTRODUCTION

The process of blood cell formation, termed haemopoiesis, is one of the most complex examples of multilineage differentiation, resulting in the production of at least nine functionally and morphologically distinct mature cell types. The majority of these mature blood elements are short lived and must be continuously replenished from a small population of pluripotential stem cells. As some indication of the enormity of this process, simply to maintain circulating myeloid cells at normal steady state levels, an average human must produce about 3.7×10^{11} cells every day of adult life (Dexter,1987). Furthermore, the haemopoietic system must retain a degree of flexibility allowing elevated production of particular cell types in reponse to stress situations such as blood loss or infection. Clearly there must be stringent controls on this process to ensure that a stable balance throughout the different cell compartments is maintained. This chapter will review the literature available up until the end of 1986, on mouse haemopoietic cell differentiation and the growth factors involved in its regulation. IL-3, the subject of this thesis, will be discussed in some detail including comparisons with IL-3 of other species. More recent literature is reviewed in the General Discussion (Chapter 8) of this thesis.

ORGANISATION OF THE HAEMOPOIETIC SYSTEM

HAEMOPOIETIC STEM CELLS

Haemopoietic stem cells arise early in embryogenesis and are first found in the yolk sac. Later these cells migrate to the fetal liver and then on to the bone marrow - the major site of haemopoiesis during adult life. Stem cells are characterised by their extensive proliferative potential and their ability to generate daughter cells of equivalent potential (self-renewal). As stem cells differentiate they give rise to progenitor cells of more restricted differentiation and self renewal capacity. The most primitive cell in the heirarchy is the pluripotential haemopoietic stem cell (PHSC) able to give rise to both myeloid and lymphoid progeny. The existence of such cells, capable of long-term engraftment of lethally irradiated recipients, was first demonstrated using radiation-induced chromosomal markers showing conclusively that precursor cells from several lineages (myeloid and lymphoid) carried the same karyotype marker (Abramson et al.,1977). More recently an elegant experiment, using retroviruses as markers to follow the progeny of single stem cells, has defined a predominant population of stem cells with both myeloid and lymphoid potential (Lemischka et al.,1986). The self renewal and differentiation of PHSCs must be tightly regulated since these cells represent the ultimate source of haemopoietic cells that must persist throughout life.

Pluripotent stem cells have yet to be isolated and defined by surface characteristics but functional assays have been described

which allow quantitation of cells with self-renewal potential and extensive proliferative capacity. Till and McCulloch (1963) transplanted haemopoietic cells into lethally irradiated mice and showed that some of the cells lodged in the spleen where they formed macroscopic nodules. Subsequent work revealed that these nodules arise from single cells (Becker et al.,1963). Since each colony may contain upwards of a million cells, this implies an extensive proliferative potential of the colony forming cell. Furthermore, the nodules, derived from a single cell, contained multiple cell lineages (Curry and Trentin,1967). A small number of these spleen colonies contained cells capable of producing more spleen colonies when transplanted into secondary irradiated recipients indicating that spleen colony forming cells (CFU-S) possess some self renewal capacity (Siminovitch et al.,1963, Cudkowicz et al.,1964).

There appear to be 2 subclasses of CFU-S. Spleen colonies measured 7 - 8 days after transplantation (CFU-S 7/8) are predominantly composed of cells of a single lineage and do not contain cells capable of spleen colony formation in secondary hosts. In contrast, spleen colonies harvested 12 - 14 days after transplantation (CFU-S 12/14) contain multiple cell lineages and cells that are capable of secondary spleen colony formation (Magli et al.,1982). The self renewal capacity of CFU-S (12/14) together with their apparent lack of sensitivity to drugs cytotoxic for actively cycling cells (Zant,1984; Hodgson and Bradley,1979) suggests that these cells are the more primitive of the CFU-S populations. It is generally agreed however that neither CFU-S population comprises truly primitive stem cells but represent a

heterogenous population including multipotent stem cells and more mature cells which have lost much of their capacity for self renewal (Hodgson and Bradley,1979; Magli et al.,1982).

During attempts to assay stem cells *in vitro* in clonogenic semi-solid culture systems, a primitive haemopoietic progenitor cell, the 'S-cell', has been described The S-cell forms colonies containing undifferentiated blast cells. Cells from these colonies can generate secondary blast cell colonies or colonies containing multiple myeloid lineages when replated (Nakahata and Ogawa,1982). S-cell colonies also contain cells which can give rise to spleen colonies indicating that the S-cell may be more primitive than the CFU-S population. As with PHSC and CFU-S(12/14), the S-cell is relatively resistant to drugs cytotoxic for actively cycling cells, indicating that it is not actively cycling *in vivo*, but the precise relationship between the PHSC, S-cell and CFU-S is not clear.

COMMITTED CELLS

Committed progenitor cells are identified by the progeny they produce. The multipotential myeloid stem cell [CFU- granulocyte, erythrocyte, monocyte, megakaryocyte (CFU-GEMM or CFC-Mix)] forms colonies containing multiple myeloid lineages and exhibits some self-renewal capacity. There is a significant correlation betweeen the number of secondary spleen colonies and the number of multilineage colonies obtained from primary spleen colonies indicating that CFU-GEMM and CFU-S may represent overlapping

populations (Johnson,1980). Unlike S-cell colonies, replating of primary CFU-GEMM colonies results in no secondary S-cell colonies and only low frequencies of secondary CFU-GEMM colonies. This heterogeneity in self-renewal capacity suggests that CFU-GEMM probably represents a continuum of stem and progenitor cells in which the capacity for self-renewal is progressively lost as the cells become lineage restricted.

Lineage restricted progenitor cells are committed to one or two lineages. Again, these cells are not morphologically recognisable and are therefore classified on the basis of functional assays. Two classes of erythroid progenitor cells have been identified. The erythroid burst forming unit (BFU-E) forms multifocal colonies containing proliferating and differentiating erythroid cells in semi-solid culture. The erythroid CFU forms single small colonies of hemoglobinised red cells and is thought to be the immediate progeny of BFU-E (Iscove,1978). Similarly the megakaryocyte BFU (BFU-Mk) forms multi-focal colonies and is thought to represent the predecessor of the megakaryocyte colony forming unit (CFU-Mk) (Long et al.,1985). Other lineage restricted progenitor cells include the eosinophil colony forming cell (Eos-CFC), basophil colony forming cell (Bas-CFC), and the granulocyte/macrophage colony forming cells (GM-CFC) which generate granulocytes and/or macrophages, depending on the growth stimulus used (see below).

Sensitivity to drugs cytotoxic for cycling cells reveals that, even under steady state conditions, committed progenitor cells are predominantly a dividing population (Boswell et al.,1984).

MORPHOLOGICALLY RECOGNISABLE PRECURSORS

Within each cell lineage there is a documented cell sequence by which the earliest identifiable precursor cells develop into more mature progeny. Progression through this differentiation pathway is accompanied by loss of proliferative capacity and acquisition of functional properties of the mature cells.

IN VITRO MODELS OF HAEMOPOIESIS

Under appropriate conditions, adherant cells derived from the bone marrow will support the proliferation and differentiation of CFU-S for many months (Dexter et al.,1977). These long term bone marrow cultures generate progenitor cells for all the myeloid lineages as well as functionally mature myeloid end cells such as granulocytes and macrophages. By altering the constituents of the culture medium, such cultures can also be induced to form B-lymphocytes (Whitlock and Witte,1982). The maintenance of haemopoiesis in these cultures is absolutely dependent on the intimate contact between haemopoietic cells and marrow stromal cells (Dexter,1982).

GROWTH FACTORS

With the advent of semi-solid culture systems allowing the proliferation and differentiation of progenitor cells *in vitro*, it became apparent that growth of these cells required the addition of

certain factors. Such factors were found in media conditioned by the growth of various tissues and tumour cell lines. These factors were originally referred to either as colony stimulating factors (CSFs) - to denote the capacity to stimulate precursor cells to produce colonies of progeny cells - or by more precise operational terms according to the primary assay by which they were characterised. By the early 1980s large numbers of activities had been described resulting in a confusing array of names. However, improved biochemical analysis of the various complex factor sources revealed that many activities were refractory to biochemical separation and could probably be attributed to the same substance. During the last 5 years, the study of haemopoietic growth factors has advanced at a phenomenal pace and the genes encoding many of these molecules have been cloned and expressed. This has allowed the definitive assignment of particular functions to defined molecules and has whittled down the array of 'factors' to a more manageable number.

Table 1 lists seven mouse growth factors active on haemopoietic progenitor cells, the other names each factor is known by and the investigators responsible for cloning the cDNA for each factor. The growth factors are glycoproteins, active at extremely low concentrations (10^{-11} - 10^{-13}M) and exhibit a number of activities in addition to their mandatory role in stimulating haemopoietic cell proliferation. Thus, they promote the *in vitro* survival and development of the appropriate target cells and, in some cases, they stimulate important biological functional activities of mature cells.

Table 1 **Mouse haemopoietic growth factors**

Growth Factor	Other acronyms	Ref
Granulocyte-Macrophage CSF (GM-CSF)	MGI-1GM	a
Interleukin 3 (IL-3)	Multi-CSF; BPA; PSF; MCGF; HCGF; E-CSF; MEG-CSF; Eo-CSF	b
Granulocyte CSF (G-CSF)	MGI-1G	c
Macrophage CSF (M-CSF)	MGI-1M; CSF-1	d
Interleukin 4 (IL-4)	BCGF; BSF-1; TCGF-11; MCGF-11	e
Interleukin 5 (IL-5)	TRF; EDF; Eo-CSF; BCGF-11; IL-4	f
Erythropoietin (Epo)		g

cDNAs for these factors were cloned by:
[a] Gough et al.(1984). [b] Fung et al. (1984); Yokota et al. (1984)
[c] Tsuchiya et al. (1986). [d] Kawasaki et al.,(1985)*
[e] Lee et al. (1986). [f] Kinashi et al. (1986)
[g] McDonald et al. (1986)
* This report refers to human M-CSF - the cloning of a cDNA encoding mouse M-CSF had not been reported at the commencement of the work described in this thesis.

INTERLEUKIN-3

Historical perspective

IL-3 was initially defined by its ability to induce the enzyme 20 alpha-hydroxysteroid dehydrogenase (20α-SDH) in spleen cells from athymic nu/nu mice (Ihle et al.,1981). At the time 20α-SDH was regarded as a unique marker for T cell maturation so this initial characterisation led to the idea that IL-3 may mediate early events in T cell differentiation. However, experiments using highly specific assays for 20α-SDH have since shown that this enzyme is not unique to T-lineage cells (Hapel et al.,1985a). Furthermore, the induction of 20α-SDH is not an activity unique to IL-3; both GM-CSF and M-CSF maintain high levels of 20α-SDH expression in normal macrophages and GM-CSF induces 20α-SDH in nu/nu splenocytes and fetal liver cells (Hapel et al.,1985). Thus, the induction of 20α-SDH, although a highly sensitive assay for haemopoietic growth factor activity, is not a specific assay for IL-3 and does not imply a role for IL-3 in T cell development.

Based on its 20α-SDH inducing activity, IL-3 was purified to homogeneity from media conditioned by WEHI-3 cells (WEHI-3 CM) and the amino-terminal sequence of the protein was established (Ihle et al.,1983). Using this purified preparation it became obvious that this factor was active in a variety of assays which were thought to detect different factors. IL-3 not only induced the expression of 20α-SDH but also induced increased histamine synthesis by bone marrow cells, permitted colony growth from

bone marrow cells in semi-solid culture and promoted the proliferation of mast cells, persisting (P) cells and the FDC-P1 cell line. Thus, IL-3 was demonstrated to have the activities associated with histamine-producing cell growth factor (HCGF), colony stimulating factor (CSF), mast cell growth factor (MCGF) P cell stimulating factor (PSF) and WEHI-3 growth factor, respectively.

Molecular and biochemical aspects of mouse IL-3

Cloning of IL-3 cDNA

The mouse cDNA for IL-3 was initially cloned by Fung and colleagues (1984). Briefly, Poly (A)+ RNA prepared from WEHI-3B cells was fractionated by sucrose density centrifugation and then translated in *Xenopus laevis* oocytes, The translates were assayed for IL-3 activity using the IL-3-dependent cell line 32D cl-23. RNA enriched for IL-3 mRNA was then used to synthesise double stranded cDNA. A cDNA clone carrying the entire coding sequence for mouse IL-3 was then identified and sequence analysis showed that it encoded a polypeptide of 166 amino acids (Fung et al.,1984).

At about the same time, Yokota et al. (1984) isolated a cDNA clone coding for the mouse mast cell growth factor (MCGF) activity. The MCGF mRNA was isolated from a concanavalin A-stimulated mouse T cell clone and cloning was achieved using the pCD cloning vector which allows expression of cDNA inserts in mammalian cells. The nucleotide sequence of MCGF cDNA was identical with that of IL-3

cDNA except for a single nucleotide difference, thus confirming the identity of IL-3 and MCGF.

Based on the assumption that translation begins at the first methionine codon, the IL-3 polypeptide includes a hydrophobic leader sequence of 27 amino acids. Studies of the mature processed IL-3 however, are somewhat confusing. Initially, Ihle (1984) reported that the N-terminus commenced at residue 33. Further reports have identified a different N-terminus 6 residues ahead of the sequence reported by Ihle's group (at residue 27) (Clark Lewis et al.,1984; Conlon et al.,1985). The shorter form probably results from proteolytic cleavage (Clark-Lewis et al.,1984) although it is not clear whether this type of processing occurs under physiological conditions or is due to adventitious proteolysis during purification. Assuming that mature IL-3 begins at residue 27, it would be 140 amino acids long with an expected Mr of 15,674.

Comparison of the predicted amino acid sequence of IL-3 with the sequences of GM-CSF (Gough et al.,1984,1985), IL-2 (Taniguchi et al.,1983) and interferon-γ (Gray et al.,1982) has failed to detect any significant sequence homology.

Structure of the IL-3 gene

The mouse gene has been cloned and sequenced (Campbell et al.,1985; Miyatake et al.,1985), revealing a number of features of interest. The gene is composed of four introns and five exons and

the sequence of the exons is in exact agreement with that determined for the IL-3 cDNA. Intron 2 contains a tandemly repeated sequence made up of 12 similar 14 bp units with a consensus sequence AGCTCTCACCTCCC (Campbell et al.,1985). These 14 bp repeats share strong homology with a series of 20 bp repeats in the human genome which show some enhancer-like activity. The mouse IL-3 gene also has a very G-C rich region just upstream of the putative TATA box.

IL-3 genes from other species

For some time the human gene for IL-3 proved elusive. A number of laboratories tried unsuccessfully to isolate the human IL-3 gene by cross-hybridisation with its mouse counterpart. This approach did however, prove successful for isolating the rat IL-3 gene (Cohen et al.,1986). This gene was cloned and sequenced, then expressed in monkey COS cells, thus providing recombinant material for study of the biological activities of rat IL-3. A comparison of the sequences of rat and mouse IL-3 revealed that these genes had evolved significantly away from each other resulting in a sequence homology of only 54% at the protein level.

Towards the end of 1986, Yang and associates reported the identification of the human IL-3 gene. Using expression cloning, this group identified an IL-3 cDNA from a gibbon T cell line. The gibbon cDNA was then used as a hybridisation probe to isolate the equivalent human IL-3 gene. As predicted from the difficulties encountered with the cross-hybridisation approach, the sequence of

human IL-3 gene displays unusually low homology with its mouse counterpart (29% at the amino acid level).

Notably, whilst there is low homology between the coding regions of the mouse, rat and human IL-3 genes, there is a striking homology between the exon structures of these genes, suggesting that they may have a common evolutionary origin (Cohen et al.,1986; Yang et al.,1986)

Cellular sources of IL-3

The predominant physiological source of IL-3 appears to be antigen- or mitogen- activated T cells, particularly the Lyt2$^-$ L3T4$^+$ subpopulation which is reponsible for the majority of lymphokine production (Miller and Stutman,1982; Kelso and Metcalf,1985). Mosmann and associates (1986a) have proposed that this subpopulation can be further divided into two major groups: Type 1 T helper cells produce IL-2, IFN-γ, GM-CSF, and IL-3, whereas type 2 T helper cells produce GM-CSF, IL-3, IL-4 and IL-5. However, the biological significance of these proposed subclasses is obscure. Consistent with an activated T cell source for IL-3, an IL-3-like activity was found in the serum of mice undergoing severe graft versus host disease (Crapper and Schrader,1986) although IL-3 is not detectable in normal serum (Crapper et al.,1984).

The WEHI-3B (D$^-$) myelomonocytic cell line is most notable amongst the reported non-T cell sources of IL-3. This cell line

displays constitutive IL-3 production making it an attractive source of IL-3 and IL-3 mRNA. Analysis of DNA from WEHI-3B cells revealed a genomic rearrangement in the vicinity of the IL-3 gene due to the insertion of an intracisternal A type particle upstream of the promotor (Ymer et al.,1985). The constitutive synthesis of IL-3 by this cell line is thought to be a direct result of this rearrangement and therefore does not indicate that IL-3 production is a characteristic of normal cells from the myeloid or monocytic lineages.

Other non-T cell sources have been reported to produce an IL-3-like activity. Labastie et al. (1984) reported that organ cultures of yolk sac require only erythropoietin (Epo) as an external stimulus to undergo adult erythropoiesis whereas both Epo and IL-3 must be added to cultures of single yolk sac haemiopoietic cells to generate adult erythrocytes. This provides indirect evidence that the yolk sac itself may produce IL-3, although IL-3 has not yet been purified from this source. Epidermal cells produce a factor which induces the proliferation of the 'IL-3-dependent' cell lines, 32D and FDC-P (Luger et al.,1985). Similarly, astrocytes produce an activity which induces the proliferation of 32D cells and the expression of 20α-SDH in nu/nu splenocytes (Frei et al.,1986). However, the factor(s) detected in the latter two studies may not be IL-3 since 'IL-3-dependent' cell lines will also proliferate in response to other growth factors (see below) and numerous factors induce the expression of 20α-SDH (as discussed above). Further studies, such as Northern analysis to detect specific transcripts for IL-3, are required to demonstrate conclusively that yolk sac, epidermal cells or astrocytes produce IL-3.

Biological activities of IL-3 in vitro

Action of IL-3 on haemopoietic stem cells

A direct action of IL-3 on CFU-S has been reported by a number of groups. Early experiments by Garland and Crompton (1983), revealed that 3 hours culture with IL-3 increased the number of CFU-S in S phase as determined by tritiated thymidine killing. Later, Spivak and associates (1985) confirmed this action on cell cycle and demonstrated that IL-3 maintained the content of CFU-S in liquid bone marrow cultures for at least 4 days, whereas in the absence of growth factor the number of CFU-S declined to 10 - 15%. The effect of IL-3 on S-cells, the progenitors of blast cell colonies, has also been investigated. In these studies donor mice pretreated with 5 fluoro-uracil (5FU-mice) were used as a source of enriched populations of S-cells (Suda et al.,1983). Suda and associates found that IL-3 supported the growth of blast cell colonies from haemopoietic progenitors in semi-solid cultures of spleen cells from 5FU-mice. Serial observations of colony growth demonstrated the continuous emergence of blast cell colonies suggesting that multipotential progenitors are in a dormant cell-cycle state for varying times. Delayed addition of IL-3 did not alter the proliferative or differentiative characteristics of late emerging multipotential blast cell colonies (Suda et al.,1985). Together these observations suggest that S-cells do not require IL-3 for survival and IL-3 does not trigger S-cells into active proliferation but is necessary for their continued proliferation. As discussed earlier, the relationship of CFU-S to S-cells is not clear

although S-cells are believed to be more primitive and perhaps representative of the pluripotential stem cell.

Committed progenitor cells

In semi-solid bone marrow cell cultures IL-3 supports the proliferation and development of CFU-GEMM and committed progenitors from the granulocyte/macrophage, eosinophil, erythroid and megakaryocyte lineages (Hapel et al.,1985b;Rennick et al.,1985). Optimal production of mature erythrocytes requires the presence of erythropoietin (Goldwasser,1975). Similarly, although IL-3 supports the early growth of colonies containing progenitors of the megakaryocyte lineage, full megakaryocyte development to the platelet shedding stage requires thrombopoietin (Williams et al.,1984). Although for the most part the activities of IL-3 are restricted to myeloid cells, there have been reports that IL-3 can maintain precursors of T cells (Ihle et al.,1981) and of B cells (Palacios et al.,1984; Palacios and Steinmetz,1985). However, as yet these reports have not been confirmed in other laboratories.

At the commencement of the work described in this thesis there was a paucity of data on the biological activities of rat and gibbon IL-3 and as yet no data available concerning the biology of the human factor. However, in semi-solid cultures both gibbon IL-3 and rat IL-3 were known to support the proliferation of multiple types of haemopoietic cells including progenitors of the erythroid lineage (Cohen et al.,1986; Yang et al.,1986) suggesting that these factors have analogous biological activities to those of mouse IL-3.

Mature haemopoietic cells

IL-3 not only stimulates progenitors for the mast cell lineage but is also required for the proliferation and survival of the well-differentiated functional end cells (Clark-Lewis and Schrader,1981). Moreover, IL-3 opposes the induction of Ia antigens on mucosal mast cells by interferon γ, suggesting that it may modulate the function of these cells (Wong et al.,1984). Similarly, IL-3 stimulates the functional activation of mature macrophages in terms of morphology, phagocytic capacity and prostaglandin E (PGE) production, as well as supporting the survival and proliferation of these cells *in vitro* (Crapper et al.,1985).

Action of IL-3 on haemopoietic cell lines

A number of cell lines have been generated which are dependent on IL-3 for their growth. For example, FDC-P lines were established from long-term marrow culture suspension cells by repeated subculturing in the presence of IL-3 (Dexter et al.,1980). These cells could be maintained as permanently growing cell lines provided that IL-3 was continually present in the culture medium. When plated in soft agar, the early isolates produced colonies containing morphologically recognisable maturing granulocytic cells suggesting that FDC-P lines represented granulocytic precursor cells. This differentiative capacity has since been lost. Indeed, these cell lines have undergone a number of changes since their isolation and at least one of these lines, FDC-P1, can now be maintained equally well in IL-3 or GM-CSF (Hapel et al.,1984). FDC-

P1 cells bear the F4/80 antigen suggesting that they may be immortalised at some stage of macrophage differentiation.

The IL-3 dependent 32D cell lines were derived from cultures of C3H/HeJ bone marrow using a technique similar to that used to derive the FDC-P lines (Greenberger et al.,1983a,1983b). Cells of the 32D cl-23 line are alcian blue positive, placing them in the mast cell lineage. These cells can give rise to mast cell/basophil colonies in soft agar. Proliferation of 32D cl-23 cells is supported by either IL-3 or IL-2 (Hapel et al.,1984).

Experiments using continuous cell lines have highlighted the requirement for IL-3 for the survival of haemopoietic progenitor cells. As with normal (freshly isolated) progenitor cells, culture of 32D or FDC-P cells in the absence of growth factor leads to rapid cell death (Whetton and Dexter, 1983; Metcalf, 1985). The mechanism by which IL-3 maintains these cells has been investigated using FDC-P2 cells as a model. Cells cultured in the absence of IL-3 undergo a steady and rapid depletion in ATP levels, whereas those in the presence of IL-3 maintain a constant level of ATP (Whetton and Dexter, 1983). Furthermore, studies on the rate of hexose transport in IL-3 dependent cell lines revealed that transport is activated by the addition of IL-3 to the cells (Whetton et al.,1984). Thus IL-3 may maintain cell survival via the activation of hexose transport, glycolysis and primary metabolism.

Since IL-3-dependent cell lines repond to IL-3 in a dose dependent manner, they provide a useful basis for assays to detect and measure biologically active IL-3. Such assays involve either

measurements of the incorporation of tritiated thymidine by proliferating cells (Hapel et al.,1984) or spectrophotometric detection of dyes which stain living cells (Mosmann,1983). The availability of these sensitive assays facilitated the purification of IL-3 (Bazill et al.,1983) and molecular cloning of the IL-3 gene (Fung et al., 1984).

Biological activities of IL-3 *in vivo*

Whilst there is a range of information available on the actions of IL-3 *in vitro* it is only recently that enough purified material has been available for assessment of its activity *in vivo*. In one of the earliest *in vivo* studies, osmotic pumps containing recombinant IL-3 were placed under the skin of normal and sublethally irradiated mice, allowing the continuous infusion of large amounts of IL-3 for up to 7 days (Kindler et al.,1986). In mice with progenitor cell levels depressed by sublethal irradiation, 7 day treatment with IL-3 resulted in a return to near normal levels of these cells. In normal mice, this treatment resulted in a greater than 2-fold increase in numbers of haemopoietic progenitor cells within 3 days. This enhancement of hematopoiesis occurred primarily in the spleen and liver, although slight decreases in progenitor levels in the bone marrow were documented.

Broadly similar effects on progenitor cells were observed after a 6 day regime involving regular intraperitoneal injection of 100,000 units of IL-3 (Metcalf et al.,1986). In the latter study, up to 100 fold increases in the levels of mast cells were found along with an

increased number of morphologically identifiable precursors including granulocytes, eosinophils and nucleated erythroid cells. The most striking overall population changes were in the peritoneal cavity where large increases in numbers of macrophages, eosinophils, and neutrophils were observed, probably due at least in part to an influx of cells from the circulation. In addition, there was evidence of increased peritoneal macrophage phagocytic activity.

Exogenous administration of high concentrations of IL-3 also has a rapid and profound effect on the cycling status of CFU-S *in vivo*. Lord and associates (1986) reported that administration of IL-3 resulted in a greater than 2.5 fold increase in the number of CFU-S in cycle over a period of 2 - 6 h. In the spleen, both early and late appearing CFU-S reached 6 - 8 times control levels after 4 days of treatment with IL-3.

In an attempt to reduce the massive doses of IL-3 required for *in vivo* studies, Broxmeyer and associates (1986) carried out a series of experiments using mice pretreated with lactoferrin (LF), a material which suppresses release of some hemopoietic growth factors and inhibitors *in vivo*. This pretreatment was designed to reduce both the background of normal haemopoiesis and the masking effects of growth factor induced release of suppressor molecules, thus facilitating the detection of relatively small quantities of exogenously administered growth factors. Using this model, an infusion of 2,000 units of IL-3 was sufficient to enhance the cycling status and absolute numbers of all progenitors in the bone marrow and to a lesser extent in the spleen.

Together these experiments confirm that IL-3 has the ability to act on haemopoietic cell populations *in vivo*. However, considering the inherent complexity of *in vivo* studies it is not possible to determine to what extent the reported effects are due to the direct action of IL-3 on haemopoietic stem cells and progenitor cells as opposed to effects mediated via accessory cells in blood, spleen or marrow.

OTHER HAEMOPOIETIC FACTORS

This section will briefly review the biology of other haemopoietic factors in order to put the activities of IL-3 into a broader perspective.

Growth factors

In general haemopoietic growth factors are classified either as multilineage or lineage-restricted factors. IL-3 is an example of a multilineage factor in that it stimulates the proliferation and development of cells from all myeloid lineages. Granulocyte-CSF (G-CSF), Macrophage-CSF (M-CSF) and erythropoietin (Epo) on the other hand are more lineage restricted factors. G-CSF promotes the proliferation and development of GM-CFC into neutrophils and induces terminal granulocytic differentiation of a subline of the myelomonocytic leukemia WEHI-3B [WEHI-3B (D^+)] (Nicola et al.,1983). M-CSF acts mainly on the macrophage lineage although it

also stimulates a small percentage of granulocyte-containing colonies (Burgess et al.,1985) and acts on early progenitor cells in the presence of other factors (see later). Epo promotes the proliferation and haemoglobinisation of CFU-E (McDonald et al.,1986).

GM-CSF was initially thought to be restricted in action to GM-CFC. However, when assayed at relatively high concentrations, GM-CSF can also initiate (but not sustain) cell divisions in multipotential, erythroid and megakaryocyte precursors and is an effective proliferative stimulus for eosinophils (reviewed in Metcalf,1986). At yet higher concentrations (>800 units/ml), GM-CSF exhibits a capacity to stimulate typical megakaryocyte colony formation and the formation of mixed erythroid colonies (Metcalf et al.,1986b). Thus at high concentrations, GM-CSF apparently mimics many of the activities of the multilineage factor, IL-3.

The activities of IL-3, GM-CSF, G-CSF and M-CSF are apparently restricted to stem cells and committed myeloid progenitor cells. However, two recently defined growth factors, IL-4 and IL-5, have activities on both myeloid and lymphoid cells. IL-4 was first characterised as a growth factor for B cells but actually acts on a broader range of target cells including some mast cell lines (Lee et al.,1986; Mosmann et al.,1986b). Another B cell growth factor, IL-5, also stimulates differentiation of bone marrow stem cells into eosinophils (Sanderson et al.,1986).

A less well characterised growth factor has been isolated from long term bone marrow cultures (Lord and Wright,1980). This

factor stimulates the proliferation of CFU-S and is produced in greater amounts when stem cells are depleted from long term cultures (Toksoz et al.,1980).

Inhibitory factors

A number of factors apparently have inhibitory rather than stimulatory effects on haemopoiesis. For example, the interferons (IFN) inhibit growth factor induced mouse myeloid colony formation in semi-solid cultures (Klimpel et al.,1982; van't Hull et al.,1978) and the E series prostaglandins (PGE) have a potent inhibitory effect on monocytopoiesis (Kurland et al.,1978). Since macrophages, particularly those stimulated by M-CSF, are a source of both PGE and IFN, production of these inhibitors is proposed to act as a negative feedback mechanism limiting excessive mononuclear phagocyte proliferation (Moore et al., 1984)

Another inhibitory factor, detected in both normal bone marrow and long term bone marrow cell cultures, reversibly suppresses the proliferation of CFU-S (Lord et al.,1976; Lord and Wright,1980). This factor, together with the CFU-S stimulatory factor described in the previous section, may play an important role in the regulation of stem cell proliferation *in vivo*.

INTERACTIONS BETWEEN GROWTH FACTORS

Synergy between growth factors has been demonstrated in a number of *in vitro* systems. In the earliest studies on synergy, Iscove and colleagues (Iscove et al.,1982) reported a requirement for both burst promoting activity (BPA, IL-3) and Epo for the proliferation and differentiation of primitive red cell precursors (BFU-E). BPA also augmented macrophage colony growth in the presence of purified M-CSF. Iscove proposed that BPA synergises with M-CSF and Epo by expanding a population of pluripotent haemopoietic precursors and their early committed progeny: M-CSF or Epo act on later cells which have acquired the appropriate receptors as part of their differentiation programmes. In support of this proposal, cells responding synergistically to IL-3 plus M-CSF are enriched in bone marrow from 5-FU mice, suggesting that these cells are early progenitors (Bartelmez et al.,1985; McNiece et al.,1984). Recent studies have demonstrated that IL-3 also synergises with IL-4 to support the proliferation of mast cell lines (Mosmann et al.,1986b).

Another factor with profound synergistic activities is termed hemopoietin-1 (H-1). H-1 alone has no effect on haemopoietic cell proliferation and is therefore not strictly a haemopoietic growth factor. However, H-1 synergises with M-CSF to stimulate developmentally early cells to generate M-CSF receptor bearing cells. This synergistic activity is apparently restricted to cells more primitive than those responsive to M-CSF alone. Stanley and associates (1986) demonstrated that H-1 also synergises with IL-3. In cultures of bone marrow enriched for primitive precursors, a

greater total number of colonies and a greater proportion of colonies containing blast cells were produced in H-1 plus IL-3 than in IL-3 alone. Furthermore, a significant number of the H-1 plus IL-3 induced colonies could be subcultured 3 times whereas cells from IL-3 colonies displayed less proliferative capacity. This suggests that H-1 enables IL-3 to act on cells more primitive than those stimulated to proliferate and differentiate by IL-3 alone. More recent work has shown that H-1 is probably identical with IL-1 (see Introduction to Chapter 4).

MEMBRANE RECEPTORS FOR HAEMOPOIETIC GROWTH FACTORS

Radiolabelled IL-3, GM-CSF, M-CSF, G-CSF and Epo have all been shown to bind to specific high affinity cell surface receptors. The best characterised of these is the M-CSF receptor which is present predominantly on cells of the monocyte/macrophage lineage (Guilbert and Stanley, 1980; Byrne et al.,1981; Chen at al.,1984). The average number of receptors for M-CSF ranges from 3,000 - 15,000 per cell (Byrne et al.,1986). This receptor is a tyrosine-specific kinase of M_r 160,000, which autophosphorylates following the binding of its ligand (Stanley and Jubinski,1984).

Receptors for IL-3 are found on all morphologically recognisable granulocytic, monocytic and eosinophilic cells and appear to be of 2 molecular weights - 55,000 and 75,000 (Nicola and Metcalf, 1986; Palazynski and Ihle,1984). The distribution of GM-CSF receptors is similar to that of the IL-3 receptors (Metcalf,1986). Walker and Burgess (1985) reported the presence of both high and low affinity

GM-CSF receptors and determined that the size of at least one of these receptors is 51,000. However, this data was later challenged by Park et al (1986) who found exclusively low affinity receptors, M_r 130,000, on mouse bone marrow cells. The G-CSF receptor is a monomer of M_r 150,000 and is found on mouse granulocytic cells and some cells of the monocytic lineage (Nicola and Metcalf,1985; Metcalf,1986). Epo receptors are apparently restricted to erythroid progenitor cells (Krantz and Goldwasser,1984).

In contrast to receptors for M-CSF, the average number of IL-3, GM-CSF, G-CSF, and Epo receptors on normal cells is in the region of only 50-1000 per cell (Metcalf,1986; Krantz and Goldwasser,1984). Despite these low receptor numbers, half maximal proliferative effects can be achieved by G-CSF and GM-CSF with a receptor occupancy of only 5 - 10% (Metcalf,1985) and by Epo with receptor occupation in the region of 1% (Krantz and Goldwasser,1984). Degradation of M-CSF-receptor complexes appears to be very rapid (Tushinski et al.,1982) but G-CSF-receptor complexes are degraded much more slowly with a half life of at least 6 h (Nicola and Metcalf,1985). This has lead to speculation that persistence of growth factor-receptor complexes of the latter type may permit sustained response to the factor thus compensating for the low absolute number of bound receptors (Metcalf,1985).

There is no direct competition for receptor binding between IL-3, GM-CSF, G-CSF, or M-CSF but a complex set of interactions can apparently occur. Walker and associates (1985) found that IL-3 down-modulated (reduced the number available for binding) IL-3,

GM-CSF, M-CSF, G-CSF and high affinity GM-CSF receptors but had no effect on the low affinity GM-CSF receptors. Similarly, binding of GM-CSF to its receptors down-modulated its own receptor and those for G-CSF and M-CSF although it had no effect on the IL-3 receptor. High concentrations of M-CSF and G-CSF down modulated GM-CSF and M-CSF receptors respectively. Since most granulocytes and monocytes simultaneously express receptors for at least 3 of these factors (Metcalf,1986), the down-modulation effect presumably reflects the responses of individual cells following binding of the growth factor to its receptor. However, the significance of this phenomena and the apparent hierarchy of growth factors remains to be determined.

BIOLOGICAL ROLE OF IL-3

The broad range of activities of IL-3 suggests that it plays a central role in haemopoiesis. However, since IL-3 is apparently produced solely by activated T cells, many investigators have proposed that this factor is involved in immunological regulation of haemopoietic activity as distinct from constitutive (steady-state) haemopoiesis. Furthermore, in view of the overlapping activities of IL-3 and GM-CSF, IL-3 may be involved principally as a mucosal mast cell factor and as a stimulus to expand the precursor pool for cells involved in local antiparasite inflammatory responses: GM-CSF, on the other hand, may function as a mediator in inflammatory cell hyperplasia involving neutrophil and macrophage proliferation and activation following bacterial infection.

The major drawback of these proposals is that IL-3 receptors are present on a broad range of haemopoietic progenitor cells which, in the adult, are restricted mainly to the bone marrow. This is a seemingly purposeless situation since these cells are unlikely to be exposed to IL-3 produced by activated T cells - IL-3 is detected in the serum only under the conditions of widespread T cell activation caused by severe graft-versus-host disease.

An alternative viewpoint stems from studies of *in vitro* models of haemopoiesis. The observation that stromal cells can support haemopoiesis in the absence of added growth factors suggests that these cells can produce all the growth factor activity required to support haemopoietic proliferation and differentiation. Significantly however, this range of activities is not detectable in media conditioned by stromal cells from long term marrow cultures. A suggestion which accomodates both these observations is that stromal cells produce growth factors such as IL-3 at undetectably low levels, perhaps in a membrane bound form which is presented directly to developing haemopoietic cells. This model is supported by the consistent intimate cell associations seen between stromal and haemopoietic cells in long term marrow cultures.

AIM OF THIS THESIS

The biology of IL-3 is obviously complex, involving unique, overlapping and synergistic activities and at the present time

there are widely differing opinions on the role of this factor *in vivo*. Most of the literature on the biology of IL-3 is based on the results of experiments using a few inbred strains of mice. However, there is some evidence that the proliferative response of bone marrow cells to IL-3, varies according to the strain of the donor mice. The initial experiments described in this thesis were performed to determine the extent of this intraspecies variation (Chapter 2).

Bone marrow cells from two strains of mice, A/J and AKR, were found to be particularly unresponsive to IL-3. IL-3 did not support colony formation from A/J and AKR cells in soft agar cultures but supported a low level of proliferation in liquid cultures of these cells. Experiments were then designed to test a number of alternative hypotheses which would account for these observations (Chapter3).

These experiments showed that IL-3 had little or no direct mitogenic activity on haemopoietic cells from A/J or AKR mice. Haemopoietic cells from these strains of mice were then used as a novel system to analyse:

. The contribution of the direct mitogenic activity of IL-3 in the synergistic response to IL-3 plus M-CSF (Chapter 4);
. The effect of IL-3 on c-*fms* (M-CSF receptor) RNA expression (Chapter 5);
. The synergistic interactions between IL-3 and other haemopoietic cytokines, notably erythropoietin, IL-1 and IL-4 (Chapter 6); and

. The effect of IL-3 on the synthesis of other haemopoietic growth factors in cultures of bone marrow cells.

Chapter 2

Strain-dependent variation in the proliferative response to IL-3

INTRODUCTION

The biology of mouse IL-3 has been extensively studied *in vitro* and it has been shown that IL-3 stimulates the proliferation of a broad spectrum of haemopoietic cells including pluripotential stem cells and the progenitors of granulocytes, macrophages, erythrocytes, megakaryocytes and mast cells (reviewed in Chapter 1). Most of this experimental data has been gleaned from studies using a few inbred strains of mice, particularly C57BL/6, CBA, and C3H/HeJ. However, at least two studies in which the proliferation of bone marrow cells from different strains of donor mice was compared, indicate that there are variations in IL-3-responsiveness (Kincade et al.,1979; Horland et al.,1980.). To determine the extent of such intraspecies variation, we compared the proliferative activity of IL-3 and other growth factors on haemopoietic cells isolated from a range of strains of donor mice. The two biological assays we used for this study are discussed below.

Soft agar colony assays

The first *in vitro* progenitor cell assays were independently reported by Bradley and Metcalf (1966) and Pluznick and Sachs (1965). These authors demonstrated that bone marrow cells suspended in semi-solid agar cultures in the presence of "feeder cells" or conditioned media proliferated to produce discrete colonies. This culture technique now forms the basis for *in vitro* assays used to study the characteristics of colony

forming cells and the colony stimulating factors necessary for their development.

The basic soft agar colony assay involves combining equal volumes of double strength culture media and liquified 0.6% agar to produce a liquid agar-media mix. The cell suspension to be cultured is added to the agar-media and 1ml aliquots are pipetted into 35 mm petri dishes and allowed to set. The colony stimulating factors or conditioned media to be tested are dispersed in the agar-media before gelling. After the required culture period, usually 7-10 days, colonies can be scored unstained in situ using a dissecting microscope or can be fixed and stained for identification of colony types. The colony assays are particularly useful for qualitative identification of growth factors since each growth factor stimulates the production of a different range of colony types.

In a modification of the basic technique, we set up our colony assays in 12 well leukocyte migration plates rather than 35 mm petri dishes. This allows us to perform 12 mini assays on each plate so that experiments involving large numbers of colony assays are more manageable and less costly of materials.

Liquid proliferation assays

Even with the modification used in our laboratories, colony assays are somewhat cumbersome and time consuming. Therefore, for routine detection of growth factor activity, we

have developed a 3 day micro-assay based on measurement of [^{3}H]-thymidine incorporation by proliferating cells in liquid culture. For this assay $1x10^5$ bone marrow cells are incubated in 100 µl cultures in 96 well microtitre plates. After 48 hours, the cells are pulsed with [^{3}H]-thymidine overnight (16-18 hours), subjected to one cycle of freezing and thawing and harvested onto glass fibre filters. The incorporated label can then be determined by scintillation counting.

Unlike the soft agar cultures, these assays do not provide data on the lineages of the cells stimulated. However, the liquid proliferation assays are up to 40 times more sensitive than the soft agar colony assays (Hapel, personal communication) and allow studies of progenitor cell responsiveness to growth factors to be set up both quickly and cheaply. The major disadvantage of this technique is the high density of cells required which amplifies any effect caused by endogenous growth factor or inhibitor production. In addition, because the cells are not immobilised in a semi-solid support, results may be influenced by direct cell-cell interactions which do not occur in the colony assay.

MATERIALS AND METHODS

CHEMICALS AND GROWTH FACTORS

Unless otherwise stated all chemicals were from Sigma Chemical Co., St. Louis, Mo., U.S.A. and were of analytical grade. Recombinant mouse IL-3 was obtained from Biotechnology Australia. The amount of IL-3 stimulating half maximal proliferation of FDC-P1 cells in [^{3}H]-thymidine incorporation assays (Hapel et al. 1984) was defined as 1 unit (U). Recombinant mouse GM-CSF was obtained from Immunex Corp. Seattle, USA, and affinity purified M-CSF was kindly provided by Dr R. Stanley (Albert Einstein College of Medicine, Yeshiva University, New York U.S.A.). The amount of GM-CSF or M-CSF stimulating half maximal colony numbers from 1 x 10^4 mouse bone marrow cells in 400 µl colony assays (as described in method 2.3) was defined as 1 U. In some assays media conditioned by L929 cells was used as a source of M-CSF. Conditioned medium was harvested from 3 day confluent cultures of L929 cells in Dulbecco's modified Eagle's medium (DMEM; Gibco). All materials used were endotoxin free as determined by the Limulus amebocyte lysate assay (ACC Inc., MA, USA).

METHODS

2.1 Bone marrow cell preparation

A-TL and A-TH mice were obtained from the Walter and Eliza Hall Institute of Medical Research, Melbourne. All other mice were obtained from the Animal Breeding Establishment, JCSMR, ANU. Age and sex matched mice in the range of 8-15 weeks were killed and their femurs were removed by dissection using alcohol sterilized instruments. RPMI-1640 (CSL) containing 10% fetal calf serum(FCS) (Hybriserum,CSL) was forced through femur shafts using a syringe fitted with a 23 gauge needle. Marrow plugs were collected in a sterile plastic tube and converted to a dispersed cell suspension by gently aspirating up and down approximately 20 times using a 5 or 10 ml pipette. Viable nucleated cell counts were determined on the basis of trypan blue exclusion using a hemocytometer. Cells were then pelleted at 200 x g for 10 mins and resuspended to the required cell density in RPMI containing 10% FCS (RPMI-10% FCS).

2.2 Foetal liver cell preparation

Foetal livers were removed from AKR, A/J and DBA/2 mice at 14 days gestation using alcohol sterilised dissecting instruments. Livers were disaggregated in RPMI-10%FCS by gently aspirating them up and down using a pasteur pipette. Cells were then counted and resuspended to the required cell density as in method 2.1.

2.3 Bone marrow colony assays

Colony assays were performed using 12 well leukocyte migration plates (Sterilin). Each plate was placed in a petri dish and the required growth factors were pipetted into each well. Saturating doses of all growth factors (determined by titration) were used except where otherwise stated. Total volume of growth factors used for each well was usually between 10 and 80 µl. For each plate (12 wells), 6 mls of 0.33% agar medium and cell suspension was prepared as follows. 1.32% agar (Difco Laboratories Inc.) was melted by heating in a microwave oven and allowed to cool to 40°C in a water bath for 1-3 hours before use. Double strength RPMI-1640 (1.5 mls) was mixed with 1.5 mls of melted 1.32% agar,1.8 mls of FCS and 0.6 mls of 10% BSA in RPMI-1640 supplemented with 0.04 volumes of 25 µm 2-mercaptoethanol (BDH Chemicals Ltd.) in 5% $NaHCO_3$. This mixture was then held at 37°C until the addition of 0.6 mls of bone marrow cell suspension at 2.5 x 10^5 cells/ml. Aliquots (0.4 mls, containing 1 x 10^4 cells) of the cell suspension in agar medium were pipetted into each well using a gentle stirring action to mix in the growth factors. To prevent the cultures from drying out, a small volume of water - sufficient to cover the base of the dish but not to wet the cultures - was added to the petri dish. The cultures were allowed to gel at room temperature, then incubated at 37°C in a humidified 5% CO_2 atmosphere. Colonies (>50 cells) were scored after 7 days using a stereo dissecting microscope.

2.4. Bone marrow cell liquid proliferation assays

In liquid culture bone marrow cell assays, incorporation of [^{3}H]-thymidine was used as a measure of cell proliferation. Serial two-fold dilutions of the sample to be tested were incubated with $1x10^5$ freshly isolated bone marrow cells in 100 µl of RPMI-10% FCS in 96 well microtitre plates (Nunclon). The starting concentrations (concentrations of factors in the first wells) of the growth factors used were as follows: IL-3, 250 U/ml; GM-CSF, 50 U/ml; M-CSF, 200 U/ml. Assays were incubated for 48 hours in a humidified, 5% CO_2 atmosphere. Cultures were pulsed for 18 hours using 0.5 µCi [^{3}H]-thymidine and subjected to one cycle of freezing and thawing before harvesting onto glass fibre filters (Titerteck) using a Titerteck cell harvester. Incorporated label was determined by scintillation counting.

2.5 Foetal liver cell liquid proliferation assays

Assays were carried out essentially as described in method 2.4 except that foetal liver cell assays were incubated for 72 hours prior to pulsing and harvesting.

RESULTS

Strain-dependent differences in the response of bone marrow cells to IL-3

A. COLONY ASSAYS

Freshly isolated mouse bone marrow cells (method 2.1) from a range of strains were plated in soft agar cultures with IL-3 or M-CSF (method 2.3) and the number of colonies produced was scored 7 days later. The number of colonies supported by M-CSF was similar regardless of the strain of the donor mouse (table 2.1). However, there was considerable strain-dependent variation in the proliferative response of bone marrow cells to IL-3. Bone marrow cells from AKR and the A-derived strain, A/J, did not produce colonies in response to IL-3 although they responded normally to M-CSF. In occasional assays, numerous small clusters, usually consisting of <20 cells, were produced by A/J and AKR bone marrow cells in the presence of IL-3

Search for linkage between IL-3 responsiveness and genes within the H-2 complex

This experiment was designed to analyse whether the low IL-3 response detected in A/J and AKR mice is controlled by a gene(s) within or linked to the H-2 complex. The response to IL-3 of bone marrow cells from strains with identical

Table 2.1 Effect of IL-3 and M-CSF on colony formation by bone marrow cells from different mouse strains

	NUMBER OF COLONIES	
MOUSE STRAIN	IL-3	M-CSF
C57Bl/6	23 ± 4	23 ± 6
BALB/c	11 ± 1	18 ± 2
C3H/HeJ	31 ± 8	NT
A/J	0	18 ± 4
AKR	0	18 ± 4

Bone marrow cells were plated on colony assays with IL-3 (200U/ml) or M-CSF (200U/ml). Data represents the mean ± SD of the number of colonies in triplicate cultures each containing 1×10^4 cells. NT, not tested.

haplotypes to those of A/J or AKR was assayed. Congenic strains were also tested to allow more accurate localisation of any linkage found. Bone marrow cells from each strain were cultured in soft agar (method 2.3) with IL-3 and the number of colonies produced was scored 7 days later. Cultures with M-CSF were set up in parallel as controls. Bone marrow cells from AKR, A/J and the congenic strains A.TH and A.TL failed to produce colonies in response to IL-3. In contrast, IL-3

supported colony formation from CBA (same H-2 haplotype and Tla type as AKR) and from B10.A (same H-2 haplotype and Tla type as A/J) bone marrow cells (Table 2.2). Therefore, H-2 and H-2-linked loci are not apparently involved in IL-3-responsiveness. Since both A/J and AKR mice are C5 deficient, the possibility that C5 deficiency is linked with low IL-3 responsiveness was also tested. Bone marrow cells from DBA/1, another C5 deficient strain, were assayed for response to IL-3 in colony assays as above. In contrast to A/J and AKR bone marrow cells, DBA/1 cells produced colonies in response to IL-3 indicating that C5 deficiency is not correlated with IL-3 response.

B. LIQUID PROLIFERATION ASSAYS

Freshly isolated mouse bone marrow cells (method 2.1) from a range of strains were plated in liquid proliferation assays with serial dilutions of IL-3 (method 2.4). The results of these assays reveal the same general pattern of strain-dependent IL-3-responsiveness observed in the colony assays (Fig 2.1). However, in this assay system bone marrow cells from A/J and AKR proliferated to a limited extent in the presence of IL-3. Further liquid proliferation assays were set up using DBA/2, BALB/c, and A/J bone marrow cells in the presence of IL-3, GM-CSF, or M-CSF. A/J and BALB/c bone marrow cells were indistinguishable in their response to both M-CSF and GM-CSF (Table 2.3). However, IL-3 induced a much greater response in BALB/c than in A/J bone marrow cells. The maximum [^{3}H]-

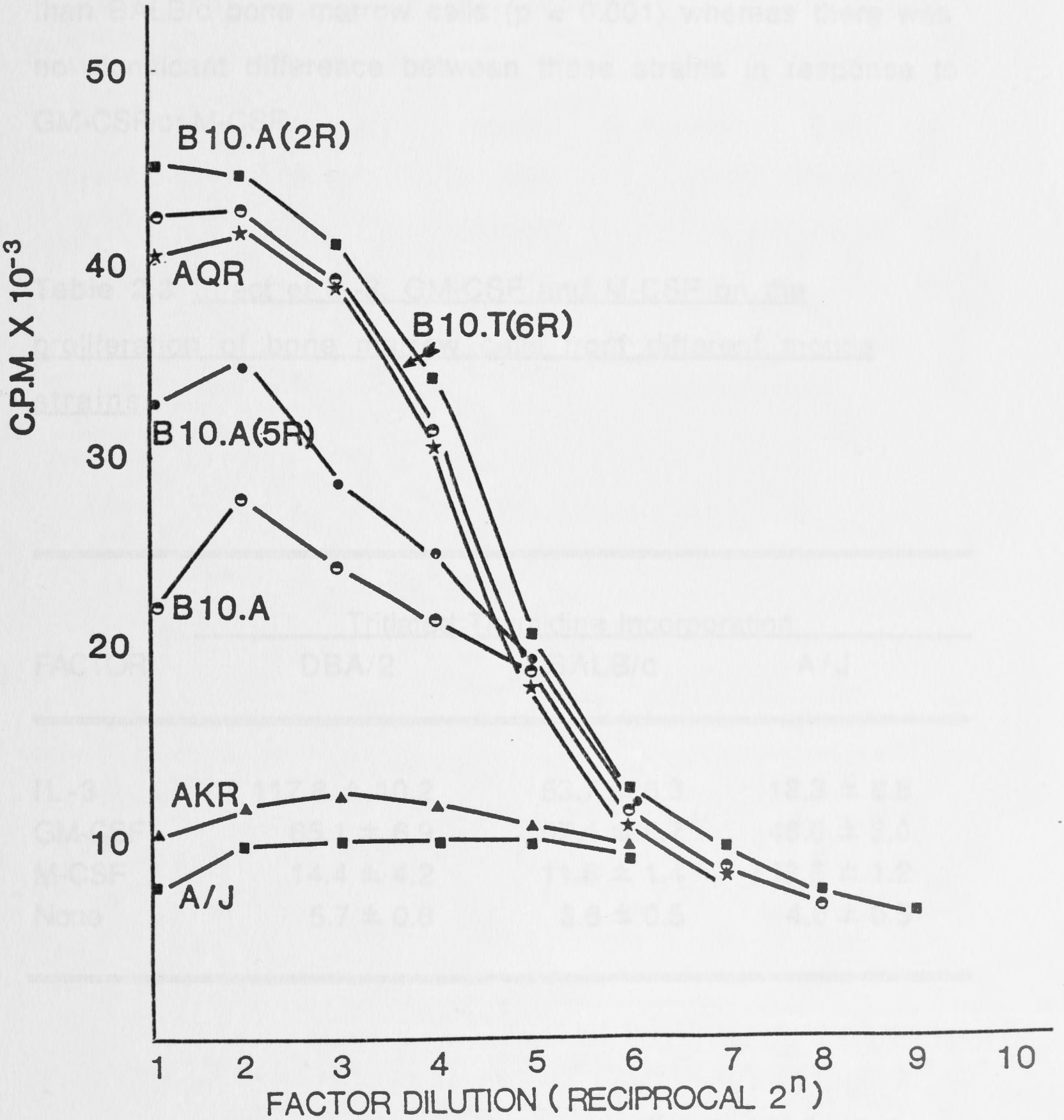
Table 2.2 Effect of H-2 haplotype and Tla type on the response of bone marrow cells to IL-3

STRAIN	H-2 Haplotype	Origin of H-2 Regions K	I	D	Tla type	Colony Number IL-3	M-CSF
DBA/1	q	q	q	q	b	37	20
CBA	k	k	k	k	b	28	NT
B10.A	a	k	k.d	d	a	19	9
B10.A(2R)	h^2	k	k.d	b	b	30	15
B10.A(5R)	i^5	k	b.k.d	d	a	26	16
B10.T(6R)	y^2	q	q	d	a	29	27
A/J	a	k	k.d	d	a	0	20
A.TH	t^1	s	s	d	a	0	22
A.TL	t^2	s	k	d	c	0	21
AKR	k	k	k	k	b	0	16

Bone marrow cells were plated in colony assays with saturating doses of IL-3 (200U/ml). Assays containing M-CSF (200U/ml) were set up in parallel. Data represents the mean of the number of colonies in triplicate cultures each containing $1x\ 10^4$ cells. NT, not tested

Fig 2.1 Effect of IL-3 on the proliferation of bone marrow cells from different mouse strains

Bone marrow cells from a range of mouse strains were cultured in serial two-fold dilutions of IL-3 (starting concentration, 250U/ml) for 2 days (method 2.4) and then pulsed for 18 h with [^{3}H]-thymidine. Results represent the mean uptake of [^{3}H]-thymidine in triplicate assays.


50
40
30
20
10
C.P.M. X 10^{-3}
B10.A(2R)
AQR
B10.T(6R)
B10.A(5R)
B10.A
AKR
A/J
1
2
3
4
5
6
7
8
9
10
FACTOR DILUTION (RECIPROCAL 2^n)

thymidine incorporation by bone marrow cells from these two strains in IL-3 was significantly different ($p < 0.001$) according to the Students t test. Similarly, the proliferative response induced by IL-3 was significantly greater in DBA/2 than BALB/c bone marrow cells ($p < 0.001$) whereas there was no significant difference between these strains in response to GM-CSF or M-CSF.

Table 2.3 Effect of IL-3, GM-CSF and M-CSF on the proliferation of bone marrow cells from different mouse strains

	Tritiated Thymidine Incorporation		
FACTOR	DBA/2	BALB/c	A/J
IL-3	117.8 ± 10.2	53.7 ± 6.3	18.3 ± 8.8
GM-CSF	65.1 ± 6.9	57.4 ± 8.7	46.6 ± 3.0
M-CSF	14.4 ± 4.2	11.6 ± 1.4	13.6 ± 1.2
None	5.7 ± 0.8	3.6 ± 0.5	4.0 ± 0.5

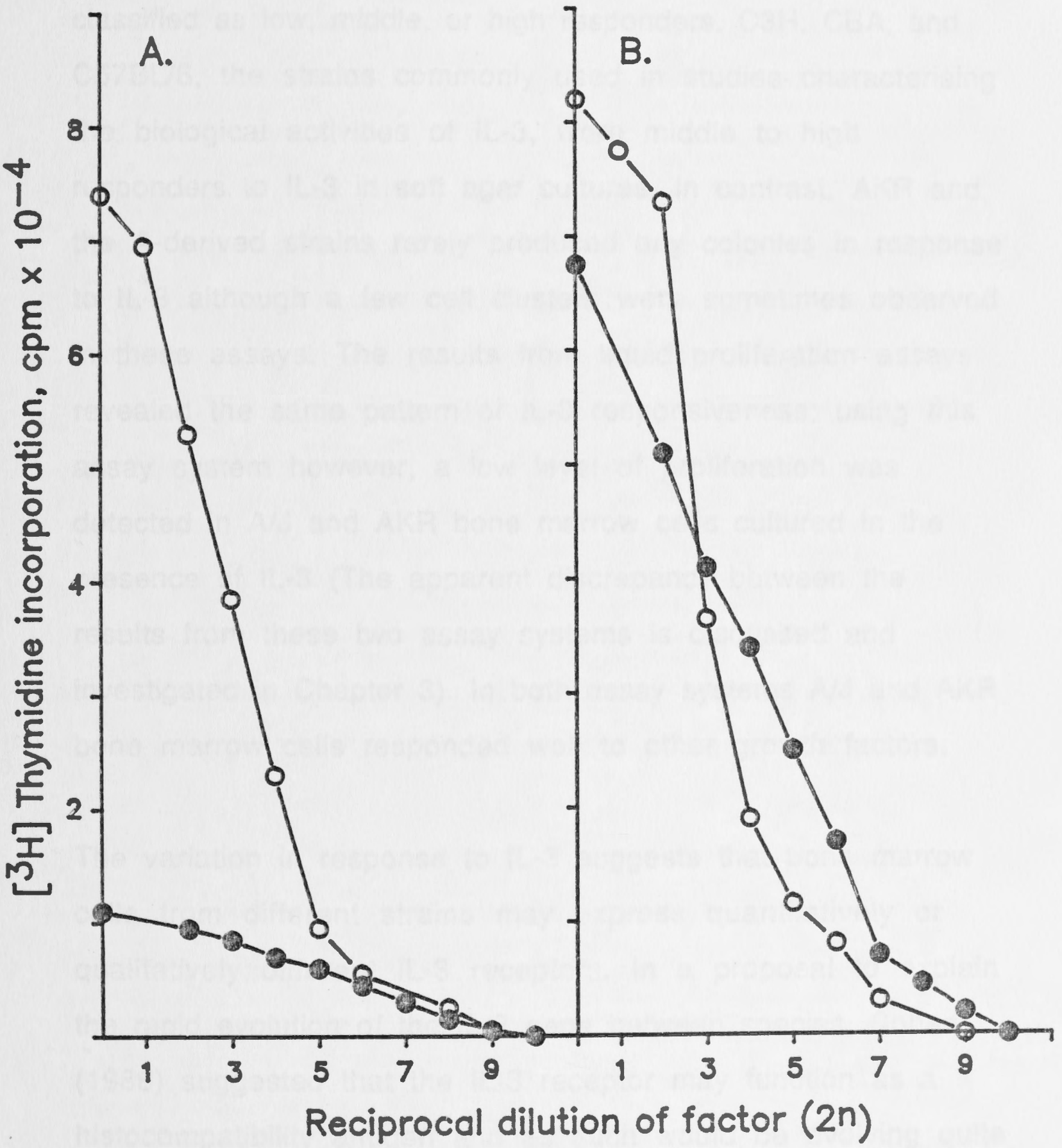
Bone marrow cells were cultured at 1×10^6 cells/ml for 2 days in IL-3 (200 U/ml), GM-CSF (40 U/ml) or M-CSF (200 U/ml). Cultures were pulsed with [^{3}H]-thymidine, harvested and counted as described in method 2.5. Data represents the mean ± SD of [^{3}H]-thymidine incorporation (cpm x 10^3) in 6 replicate wells.

Strain-dependent differences in the response of foetal liver cells to IL-3

Liquid proliferation assays (method 2.5) were also carried out using cells isolated from 14 day DBA/2, A/J, and AKR foetal livers (method 2.2) in the presence of IL-3 or GM-CSF. A/J cells responded well to GM-CSF but were relatively poor responders to IL-3 (Fig 2.2). Similar results were obtained with AKR cells (data not presented). DBA/2 cells however, responded well to both IL-3 and GM-CSF.

Fig 2.2 Effect of IL-3 and GM-CSF on the proliferation of A/J and DBA/2 foetal liver cells

(A), A/J and (B), DBA/2 foetal liver cells were cultured in serial two-fold dilutions of IL-3 (filled circles) or GM-CSF (open circles). Starting concentration of IL-3 was 250 U/ml. Starting concentration of GM-CSF was 50 U/ml. Assays were incubated for 72 h prior to an 18 h pulse with [^{3}H]-thymidine. Results represent the mean uptake of [^{3}H]-thymidine in triplicate assays.


A.
B.
[3H] Thymidine incorporation, cpm x 10−4
8
6
4
2
1
3
5
7
9
Reciprocal dilution of factor (2n)

DISCUSSION

The results of *in vitro* assays revealed that the response of bone marrow cells to IL-3 varied according to the strain of the donor mouse, such that donor strains can be arbitrarily classified as low, middle, or high responders. C3H, CBA, and C57BL/6, the strains commonly used in studies characterising the biological activities of IL-3, were middle to high responders to IL-3 in soft agar cultures. In contrast, AKR and the A-derived strains rarely produced any colonies in response to IL-3 although a few cell clusters were sometimes observed in these assays. The results from liquid proliferation assays revealed the same pattern of IL-3 responsiveness; using this assay system however, a low level of proliferation was detected in A/J and AKR bone marrow cells cultured in the presence of IL-3 (The apparent discrepancy between the results from these two assay systems is discussed and investigated in Chapter 3). In both assay systems A/J and AKR bone marrow cells responded well to other growth factors.

The variation in response to IL-3 suggests that bone marrow cells from different strains may express quantitatively or qualitatively different IL-3 receptors. In a proposal to explain the rapid evolution of the IL-3 gene between species, Cohen (1986) suggested that the IL-3 receptor may function as a histocompatibility antigen and as such would be evolving quite rapidly in line with other such antigens. This proposal would also explain the differences in response to IL-3 between mouse strains. The IL-3 receptor is unlikely to be a classical

H-2 antigen because these antigens are present on all cell types (reviewed in Mellor, 1986) whereas expression of the IL-3 receptor is probably restricted to haemopoietic cells (Nicola and Metcalf, 1986). In contrast, the related Class 1 polypeptides encoded by genes in the Qa and Tla regions of the H-2 complex have been detected primarily on cells of hemopoietic lineage (Mellor,1986). Therefore, the gene for the IL-3 receptor may be one of a number of genes in these regions which are actively transcribed but whose polypeptide products have not yet been identified. However, the data reported in this chapter indicate that the level of response to IL-3 is not correlated with H-2 haplotype or Tla type, strongly suggesting that IL-3-responsiveness is not controlled by gene(s) within the H-2 complex.

Other groups (Kincade et al.,1979; Horland et al.,1980) have reported that bone marrow cells from NZB and RF mice produce an abnormally low number of colonies in soft agar cultures containing WEHI-3B CM (a source of IL-3). In common with AKR mice, both NZB and RF mice have a high incidence of spontaneous leukaemias. Horland et al.(1980) suggested that this propensity for the development of leukaemias is linked with the low proliferative response to IL-3. Consistent with this proposal, Chevalier and co-workers (1974) showed that bone marrow cells from leukaemic AKR mice have a reduced response to a crude CSF preparation compared with age-matched non-leukaemic AKR mice. However, the abnormal IL-3 response reported here is obvious even in haemopoietic cells isolated from 14 day AKR foetal livers, whereas the onset of

leukaemias in this strain usually occurs at > 6 months of age suggesting that this disease is not directly responsible for the low IL-3 response observed. AKR and the A-derived strains are C5 deficient but this is also unlikely to be a direct cause of their low IL-3 response since another C5 deficient strain, DBA/2, is highly responsive to IL-3.

Finally, a number of studies have documented a defect in macrophages from mice of the A/J strain. For example, such macrophages do not acquire tumoricidal competence as do macrophages from other mouse strains (e.g. C57BL/6) after treatment with recombinant interferon-γ (Hamilton et al.,1986). However, further work is required to determine whether the abnormalities in IFN-γ and IL-3 responses in this strain are related.

Chapter 3

Analysis of the mitogenic activity of IL-3 on A/J and AKR bone marrow cells.

INTRODUCTION

The low levels of IL-3-induced proliferation in liquid assays of A/J and AKR cells suggested that bone marrow from these strains does contain IL-3-responsive cells. Why then were IL-3-responsive colony-forming-cells (CFCs) not detectable in soft agar assays? On the basis of the known biology of IL-3, together with the observations on the behaviour of A/J and AKR cells in liquid and soft agar cultures, the following explanations were proposed:

1. **A/J and AKR bone marrow contains an unusually small population of IL-3-responsive cells: These cells nevertheless proliferate normally in response to IL-3.** The relatively large number of cells used for each well of the liquid assay would explain why it was possible to detect the proliferation of cells comprising this proposed rare population in liquid culture but not in soft agar cultures.

2. **A/J and AKR IL-3 receptors recognise autologous IL-3 but not the BALB/c derived IL-3 used routinely in our assays.** In support of this proposal, IL-3 has evolved suprisingly rapidly between species; for example, the amino acid homology between rat and mouse IL-3 is only 54% (Cohen et al.,1986.). This low homology is reflected in low cross-species reactivity in biological assays, indicating that IL-3 and its receptor have evolved in concert. There is also evidence of strain-specific differences in the amino acid sequence of mouse IL-3 (Campbell et

al.,1987.). Therefore the IL-3 produced by A/J and AKR may have evolved significantly away from the BALB/c derived recombinant IL-3, resulting in such low cross-strain reactivity that the proliferative response is detectable only in the more sensitive liquid culture assay.

3. **A/J and AKR bone marrow cells do not possess IL-3 receptors.** If this is the case, the small proliferative response apparently induced by IL-3 in liquid cultures of A/J and AKR bone marrow cells must be an artefact caused by some mechanism other than the interaction of IL-3 with its receptor.

4. **A/J and AKR bone marrow cells do not proliferate in response to IL-3 _ the low level of proliferation observed in liquid cultures is indirectly induced perhaps via stimulation of endogenous growth factor production.** This would explain the disparity between liquid and soft agar cultures as the indirect effects would be cell density dependent and therefore most likely to be detected in the liquid proliferation assays.

5. **A/J and AKR bone marrow cells produce excessive levels of factors which inhibit haemopoietic proliferation.** Since A/J and AKR cells responded normally to GM-CSF and M-CSF, it follows that the proposed inhibitor is induced by IL-3 but not by other growth factors and/or that the inhibitor specifically antagonises IL-3-induced proliferation. Possibly, the 3

day culture period used for the liquid assays allows detection of IL-3-induced proliferation before the optimal concentration of inhibitor has been reached. The small necrotic colonies often detected in 7 day soft agar cultures of A/J and AKR cells may reflect this early proliferation prior to complete inhibition of the response to IL-3.

The experiments described in this chapter were designed to investigate each of these five possibilities. In addition, the inheritance pattern of the IL-3 responsive trait was investigated in an attempt to determine the relationship between the defects in A/J and AKR mice.

MATERIALS AND METHODS

CHEMICALS AND GROWTH FACTORS

Chemicals and growth factors used were as described in Chapter 2 unless otherwise stated. WEHI-3B conditioned medium (CM) was prepared by culturing WEHI-3B cells in RPMI-5%FCS at an initial density of 10^6cells/ml for 48 h. Radioiodinated IL-3, specific radioactivity 60,000 cpm/ng, was kindly provided by N.Nicola (The Walter and Eliza Hall Institute of Medical Research, Victoria)

METHODS

3.1 Bone marrow cell preculture

Freshly isolated bone marrow cells (method 2.1) were plated at $1x10^6$/ml in 5 ml RPMI-10%FCS in the presence of IL-3 (200U/ml) in 35 mm diameter wells (Costar). Bone marrow cells were plated without added growth factors as a control. Cultures were incubated at 37°C in a humidified 5% CO_2 atmosphere. After 48hrs, non-adherent cells were harvested and then washed extensively by pelleting and resuspending 3 times in 50ml RPMI-10%FCS.

3.2 Production of AKR spleen cell conditioned medium

Spleens were removed from 3 AKR mice, flushed with 10 mls of RPMI-5%FCS and minced using curved scissors. Single cell suspensions were prepared by repeated pipetting and the majority of the debris were removed by passing the resulting cell

suspension through a fine mesh sieve. Cells were incubated at $1x10^6$/ml in 2µg/ml concanavalin A (Pharmacia) in RPMI-5%FCS at 37°C in a humidified 5% CO_2 atmosphere; after 48 hours the conditioned medium (CM) was harvested and centrifuged at 400 x g for 15 mins to remove cells and debris. The CM was then concentrated 5 times using an Amicon stirred cell concentrator (Molecular weight cut-off,10 kd; Amicon Corp.) and dialysed against 0.01M phosphate buffer pH 7.2 (starting buffer).

3.3 Partial purification of AKR IL-3

IL-3 was partially purified from the CM on the basis that in low salt buffer at neutral pH, IL-3 does not bind to DEAE-Sephacel and can thus be rapidly separated from the bulk of the protein including GM-CSF (Ihle et al.,1981). The concentrated and dialysed CM was loaded onto a DEAE-Sephacel (Pharmacia) column which had been equilibrated with starting buffer. The unbound proteins were then eluted in the run through fractions with starting buffer. The remaining material was eluted with 0.01M phosphate buffer + 0.03M NaCl. 2ml fractions were collected, dialysed and assayed for growth promoting activity on the IL-3-responsive cell lines, 32D cl-23 and FDC-P1 (method 3.4). Peak IL-3 containing fractions were combined, concentrated 5 times using an Amicon B15 concentrator, dialysed against PBS and stored at 4°C prior to assay.

3.4 32D cl-23 and FDC-P1 proliferation assay

The factor dependent cell lines 32D cl-23 and FDC-P1 were maintained in RPMI-5%FCS supplemented with 30% WEHI-3B CM. Immediately before use, the cells were washed 3 times by pelleting at 200 x g for 10 min and resuspending in 50 ml fresh RPMI-5%FCS. Assays were performed by incubating serial two-fold dilutions of the sample to be tested with 2 x 10^4 washed cells in 100 µl of RPMI-10%FCS in 96 well microtitre plates (Nunclon) (Hapel et al.,1984.) After overnight incubation at 37°C, cultures were pulsed with 0.5 µCi [^{3}H]-thymidine for 6 hours before harvesting cells onto glass fibre filters. Incorporated label, determined by scintillation counting, was used as a measure of cell proliferation. Proliferation of 32D cl-23 cells indicates the presence of IL-3 or IL-2; proliferation of FDC-P1 cells indicates the presence of IL-3 or GM-CSF (Hapel et al.,1984).

3.5 Binding of radioiodinated IL-3 to bone marrow cells

For single time point analysis of binding of [^{125}I]-IL-3 to bone marrow cells, cells were prepared from A/J, BALB/c and C57Bl mice (method 2.1). 1 x 10^7 bone marrow cells were incubated in a total volume of 65 µl of RPMI-10%FCS in 20 mM Hepes pH 7.4 with 90,000 cpm [^{125}I]-IL-3 with or without 750ng/ml unlabelled IL-3 for 20 min at 37°C. The cell suspensions were layered over 200 µl FCS in small flexible centrifuge tubes (0.5 ml) on ice and centrifuged at 700 x g for 5 min at 4°C. The supernatant was removed by aspiration and discarded. The tips of the tubes containing the pelleted cells were then cut with a scalpel blade

and the pellet was counted in a gamma counter. Specific binding was calculated as total binding minus that in the presence of excess unlabelled IL-3.

Analysis of binding of [^{125}I]-IL-3 to bone marrow cells at various time points was performed using A/J and DBA/2 bone marrow cells. 1.6 x 10^7 bone marrow cells in 400 µl of RPMI-10%FCS were incubated with approximately 150,000 cpm [^{125}I]-IL-3 with or without 750 ng/ml unlabelled IL-3 at 37°C. At the indicated times, 50 µl samples of the cell suspension were taken. The cells in these samples were pelleted and counted as above.

3.6 Time course of tritiated thymidine incorporation in liquid bone marrow cell proliferation assays

These assays are a modification of the single time point liquid proliferation assays described in method 2.4. For the time course, 1 x 10^5 or 1 x 10^4 freshly isolated bone marrow cells were incubated in 100 µl of RPMI-10%FCS in the presence or absence of 200 U/ml IL-3 for 2, 4, or 6 days, then pulsed, harvested and counted as described in method 2.4.

3.7 Production of A/J and DBA/2 bone marrow cell conditioned media

A/J and DBA/2 bone marrow cells were cultured at 1x10^6/ml in the presence of IL-3 (200U/ml) in RPMI-5%FCS at 37°C in a humidified 5% CO_2 atmosphere. After 48 hours the CM were harvested and centrifuged at 400 x g for 15 min to remove cells

and debris. The CMs were then concentrated 5 times using an amicon stirred cell concentrator, dialysed overnight against PBS (pH 7.4) and filtered through Amicon 0.2µm filters. Samples were stored at 4°C prior to assay.

RESULTS

In vitro selection of IL-3-responsive cells

This experiment was designed to determine whether a rare population of IL-3-responsive colony-forming-cells (CFCs) could be expanded from A/J or AKR bone marrow by preculture with IL-3. For comparison, the effect of such preculture on the population of IL-3-responsive CFCs present in DBA/2 bone marrow was also assessed. Freshly isolated cells from each strain were plated in colony assays with IL-3 (method 2.3). The remaining cells were precultured (method 3.1) for 6 days in IL-3 before plating in the colony assays. The mean number of colonies produced from 1×10^4 fresh DBA/2 cells in triplicate assays was 27. After 6 days preculture with IL-3 however, the mean number of colonies produced per 10^4 DBA/2 bone marrow cells increased to 173. In contrast, even after preculture, A/J and AKR bone marrow cells did not produce colonies in response to IL-3 suggesting that bone marrow from these strains does not contain cells which proliferate normally in response to IL-3.

Partial purification and assay of AKR IL-3

A. Partial purification of AKR IL-3

AKR spleen cell CM (method 3.2) was fractionated by ion exchange chromatography using DEAE-Sephacel (method 3.3). A total of 36 fractions were collected from the column, dialysed against PBS (pH 7.4) and assayed on 32D cl-23 and FDC-P1 cells (method 3.4). The resulting column profile revealed two peaks of FDC-P1

proliferative activity (Fig 3.1). The first peak has the characteristics of IL-3 since it was eluted using low salt buffer and had activity on both FDC-P1 and 32D cl-23 cells. The majority of the second peak of FDC-P1 activity is probably GM-CSF although a small 'blip' of activity on 32D cl-23 cells indicates that some residual IL-3 may also be present.

Column fractions with peak IL-3 activity were pooled and this pooled material was concentrated 5 times and dialysed against PBS (pH 7.4) before testing in bone marrow liquid proliferation assays.

B. Assay of AKR IL-3

Next the proliferative activity of the AKR derived IL-3 was assayed on AKR and DBA/2 bone marrow cells in liquid cultures (method 2.3). BALB/c derived rIL-3 was assayed in parallel. As shown in Fig 3.2, AKR IL-3 had little activity on AKR bone marrow cells. The activity of AKR IL-3 on both DBA/2 and AKR bone marrow cells was equivalent to approx 125 U of rIL-3.

Binding of radioiodinated IL-3 to A/J bone marrow cells

Single time point analysis of the binding of [^{125}I]-IL-3 to A/J bone marrow cells was performed (method 3.5) to determine whether these cells possess receptors for IL-3. The binding of [^{125}I]-IL-3 to BALB/c and C57Bl bone marrow cells was also analysed for comparison. A/J, BALB/c and C57Bl cells bound [^{125}I]-IL-3 (Table 3.1) although the level of [^{125}I]-IL-3 bound differed between the strains: C57Bl cells bound the most [^{125}I]-IL-3 whereas A/J cells bound the least.

Fig 3.1 Separation of AKR conditioned media (CM) by DEAE-Sephacel chromatography

CM from concanavalin A stimulated AKR spleen cells was concentrated 5 times and dialysed against 0.01M phosphate buffer (PB). Five millilitres of this concentrated CM was applied to a DEAE-Sephacel column which had been equilibrated with starting buffer. Unbound material was eluted with PB. Fractions were collected at a rate of 20ml/h with 2ml/fraction. After 22 fractions had been collected the column buffer was changed to PB + 0.3M NaCl and a further 12 fractions were collected.

The column fractions were dialysed and assayed for growth promoting activity on the IL-3-responsive cell lines, 32D cl-23 and FDC-P1 (method 3.4). 32D cl-23 (open circles) and FDC-P1 (closed circles) growth promoting activity is represented as the tritiated thymidine incorporation (cpm x 10^{-3}) stimulated by a 1 in 8 dilution of the dialysed fraction.

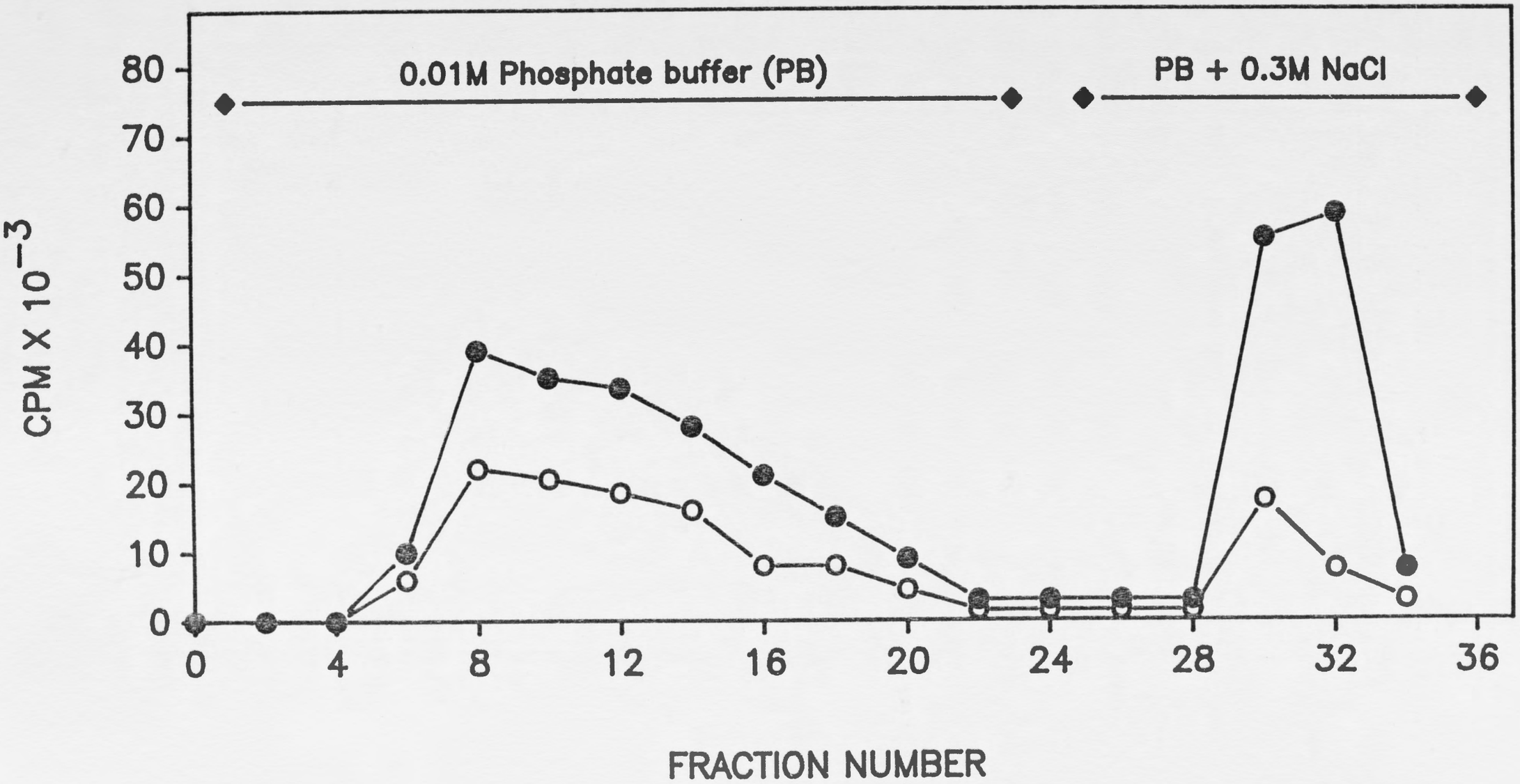

0.01M Phosphate buffer (PB)
PB + 0.3M NaCl
CPM X 10⁻³
80
70
60
50
40
30
20
10
0
0
4
8
12
16
20
24
28
32
36
FRACTION NUMBER

Fig 3.2 Proliferative activity of IL-3 isolated from AKR spleen cell conditioned medium

Dose response curves for two-fold serial dilutions of AKR IL-3 (——) and BALB/c derived rIL-3 (------) assayed against AKR (circles) and DBA/2 (diamonds) bone marrow cells (method 2.4). Preparation of AKR IL-3 is described in the text. Concentration of BALB/c derived rIL-3 was 125 U/ml. Results represent the mean uptake of tritiated thymidine in triplicate assays.

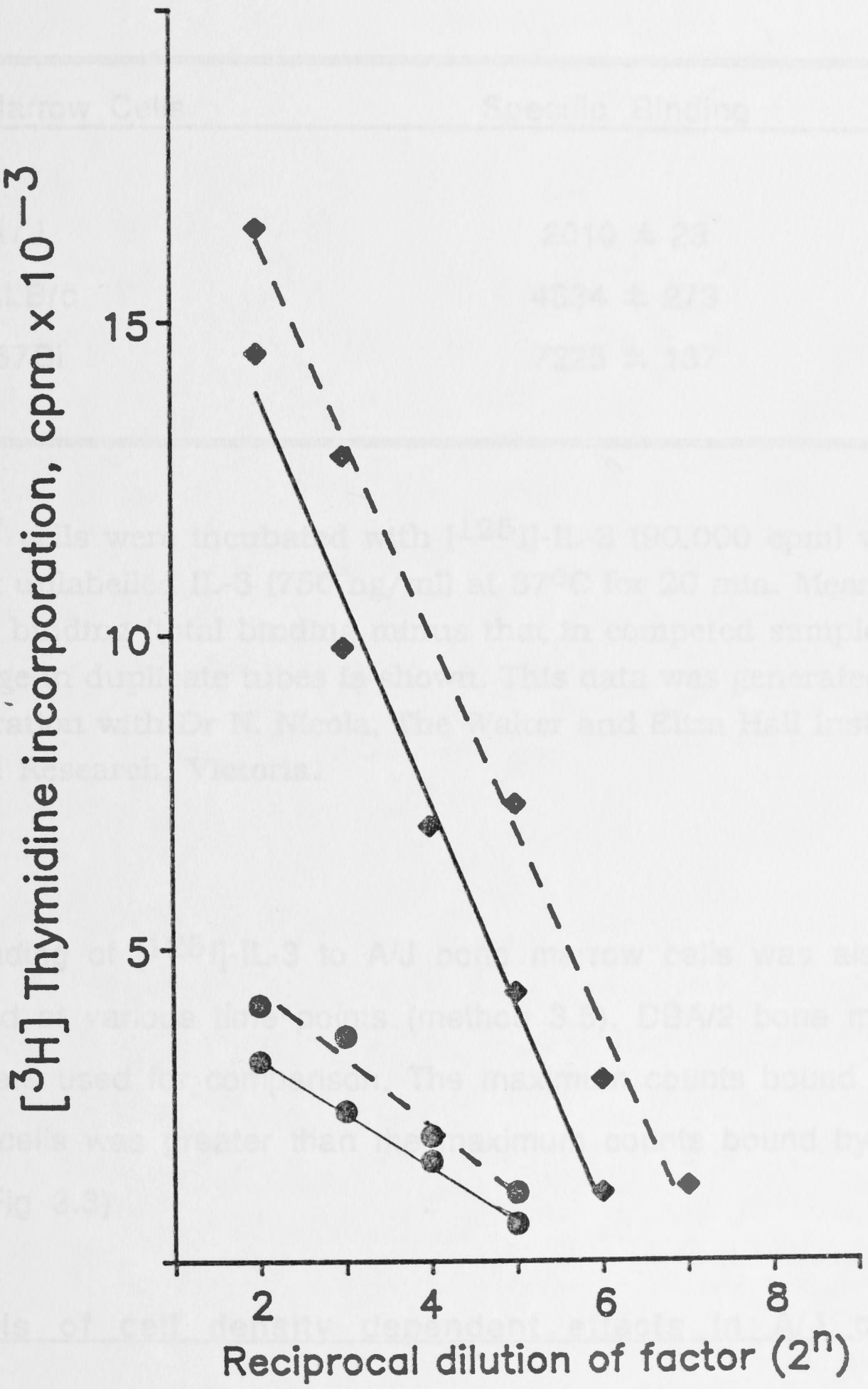

[3H] Thymidine incorporation, cpm x 10−3
15
10
5
2
4
6
8
Reciprocal dilution of factor (2n)

Table 3.1 Single time point analysis of binding of radioiodinated IL-3 to bone marrow cells

Bone Marrow Cells	Specific Binding
A/J	2010 ± 23
BALB/c	4834 ± 273
C57Bl	7225 ± 137

1×10^7 cells were incubated with [^{125}I]-IL-3 (90,000 cpm) with or without unlabelled IL-3 (750 ng/ml) at 37°C for 20 min. Mean specific binding (total binding minus that in competed samples) and the range in duplicate tubes is shown. This data was generated in collaboration with Dr N. Nicola, The Walter and Eliza Hall Institute of Medical Research, Victoria.

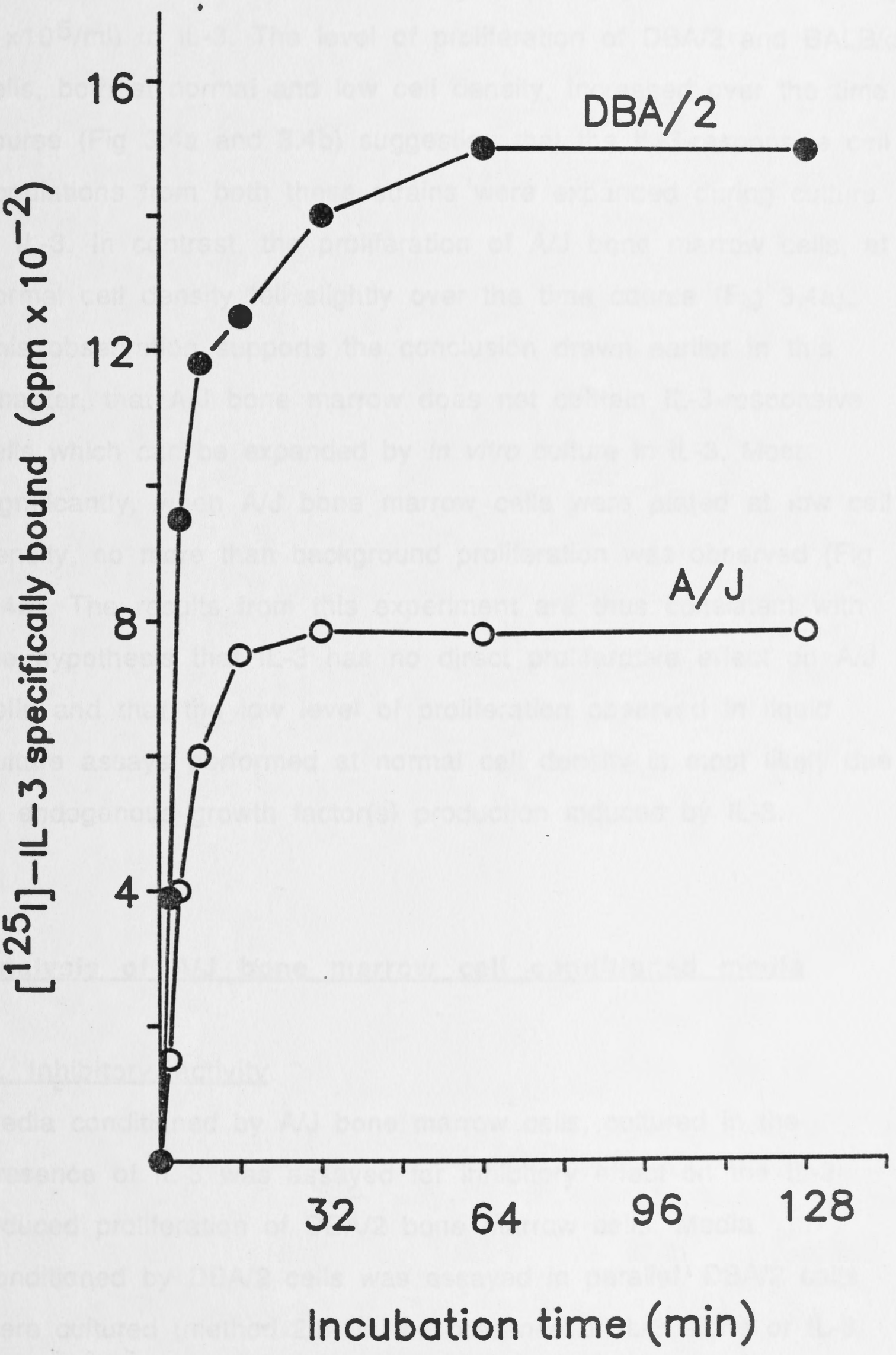
The binding of [^{125}I]-IL-3 to A/J bone marrow cells was also analysed at various time points (method 3.5). DBA/2 bone marrow cells were used for comparison. The maximum counts bound by DBA/2 cells was greater than the maximum counts bound by A/J cells (Fig 3.3)

Analysis of cell density dependent effects in A/J cultures

This experiment was designed to examine in more detail the small IL-3-induced proliferation observed in liquid cultures of A/J bone marrow cells and to analyse whether this response is directly or

Fig 3.3 Analysis of binding of radioiodinated IL-3 to bone marrow cells at various time points

A/J (open circles) or DBA/2(closed circles) bone marrow cells were incubated at 4 x 10^7/ml in volumes of 400µl with [^{125}I]-IL-3 (150,000cpm) with or without unlabelled IL-3 (750 ng/ml) at 37°C. Approximately 2 x 10^6 cells were sampled for each time point.


16
12
8
4
DBA/2
A/J
[125I]-IL-3 specifically bound (cpm x 10-2)
32
64
96
128
Incubation time (min)

indirectly induced by IL-3. A time course liquid proliferation assay was set up (method 3.6) using DBA/2, BALB/c, and A/J bone marrow cells at normal cell density ($1x10^6$/ml) or low cell density ($1x10^5$/ml) in IL-3. The level of proliferation of DBA/2 and BALB/c cells, both at normal and low cell density, increased over the time course (Fig 3.4a and 3.4b) suggesting that the IL-3-responsive cell populations from both these strains were expanded during culture in IL-3. In contrast, the proliferation of A/J bone marrow cells, at normal cell density fell slightly over the time course (Fig 3.4a). This observation supports the conclusion drawn earlier in this Chapter, that A/J bone marrow does not contain IL-3-responsive cells which can be expanded by *in vitro* culture in IL-3. Most significantly, when A/J bone marrow cells were plated at low cell density, no more than background proliferation was observed (Fig 3.4b). The results from this experiment are thus consistent with the hypothesis that IL-3 has no direct proliferative effect on A/J cells and that the low level of proliferation observed in liquid culture assays performed at normal cell density is most likely due to endogenous growth factor(s) production induced by IL-3.

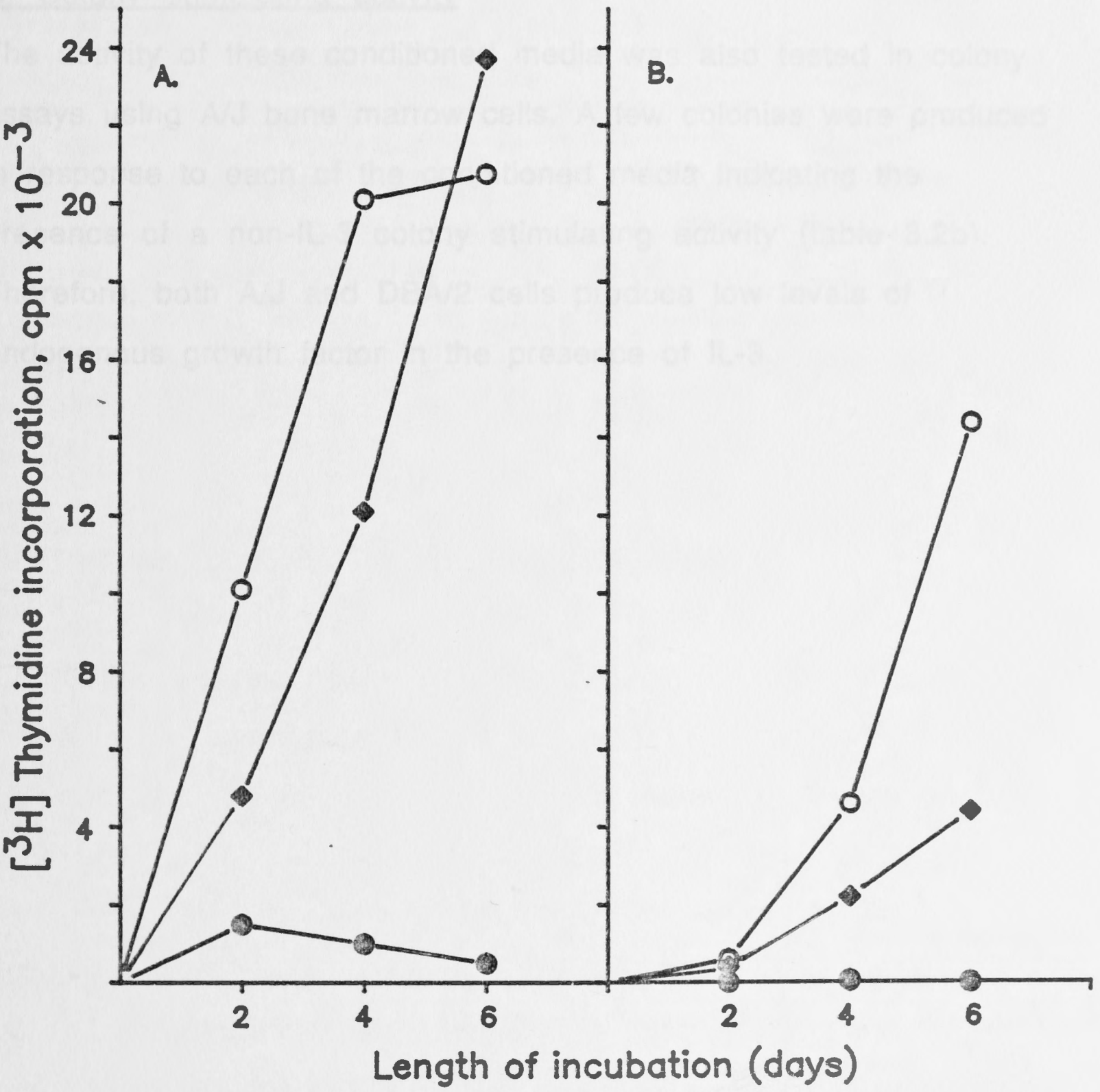
Analysis of A/J bone marrow cell conditioned media

A. Inhibitory activity

Media conditioned by A/J bone marrow cells, cultured in the presence of IL-3 was assayed for inhibitory effect on the IL-3-induced proliferation of DBA/2 bone marrow cells. Media conditioned by DBA/2 cells was assayed in parallel. DBA/2 cells were cultured (method 2.3) in the presence of IL-3 alone or IL-3

Fig 3.4 Time course of IL-3-induced proliferation in cultures of DBA/2, BALB/c and A/J bone marrow cells plated at two cell densities

Bone marrow cells from DBA/2 (open circles), BALB/c (filled diamonds) and A/J (filled circles) mice were cultured at (A) 1 x 10^6cells/ml or (B) 1 x 10^5cells/ml in 200 U/ml IL-3. After 2, 4, or 6 days the cultures were pulsed for 18 h with [^{3}H]-thymidine. Data represents the mean uptake of [^{3}H]-thymidine minus the background cpm (from cultures without added IL-3)


A.
B.
24
20
16
12
8
4
[3H] Thymidine incorporation, cpm x 10−3
2
4
6
2
4
6
Length of incubation (days)

plus one of the bone marrow conditioned media (prepared using method 3.7). Both DBA/2 and A/J conditioned media marginally reduced the colony numbers produced from DBA/2 cells in the presence of IL-3 indicating that they contained some inhibitory activity (table 3.2a). However, A/J conditioned media was no more inhibitory than media conditioned by DBA/2 cells.

B. Colony stimulating activity

The activity of these conditioned media was also tested in colony assays using A/J bone marrow cells. A few colonies were produced in response to each of the conditioned media indicating the presence of a non-IL-3 colony stimulating activity (table 3.2b). Therefore, both A/J and DBA/2 cells produce low levels of endogenous growth factor in the presence of IL-3.

Table 3.2 Colony stimulating and inhibiting activities produced by A/J and DBA bone marrow cells in culture

SOURCE OF CONDITIONED MEDIA	COLONY NUMBERS a) DBA/2	b) A/J
A/J	44 ± 8	2 ± 1
DBA/2	41 ± 1	2 ± 0
Control	53 ± 0	0 ± 0

DBA/2 or A/J cells were plated in colony assays with 200 U of IL-3 and 50 µl of the concentrated and dialysed conditioned media prepared as described in the text. The control was RPMI-5%FCS concentrated and dialysed in the same way as the conditioned media. Colony numbers are the mean ± SD of the colony counts from triplicate cultures each containing 1 x 10^4 cells.

Inheritance of A/J and AKR abnormalities

We studied the relationship between the A/J and AKR defects by following the inheritance pattern of the IL-3 responsive traits. Soft agar cultures (method 2.3) of bone marrow cells from (DBA/2 x AKR)F_1, (C_3H/HeJ x A/J)F_1, and the various parental strains were set up in the presence of IL-3. Bone marrow cells from 3 progeny of each cross were pooled. Control GM-CSF assays on each F_1 and parental strain were cultured in parallel. Bone marrow cells from (DBA/2 x AKR) and (C_3H/HeJ x A/J) F_1 mice produced colonies in response to IL-3 (Table 3.3). Further crosses between responsive and non-responsive parents consistently produced responsive

progeny (data not presented). Therefore, the responsive trait is dominant and as a corollary A/J and AKR are homozygous for the defective allelle(s) they carry.

Table 3.3 Colony stimulating activity of IL-3 and GM-CSF on F1 mouse bone marrow cells

	NUMBER OF COLONIES	
STRAIN	IL-3	GM-CSF
DBA/2	34	14
C3H/HeJ	30	13
A/J	0	17
AKR	0	20
C3H/HeJ x A/J	29	21
DBA/2 x AKR	33	16
A/J x AKR	0	19

Bone marrow cells from parental and F1 strains were cultured in soft agar on the presence of IL-3 (200 U/ml). Cultures containing GM-CSF (50 U/ml) were set up in parallel. Data represents the mean of the number of colonies in triplicate cultures each containing 1×10^4 cells.

In an attempt to assess whether the same gene(s) are defective in A/J and AKR mice the progeny of a cross between A/J and AKR were tested for their response to IL-3. If the same recessive

gene(s) are defective in A/J and AKR mice, all the progeny of a cross between these strains would be expected to be non-responsive to IL-3. However, if different genes are defective in A/J and AKR strains, the possible outcomes of a cross between these two strains is more complex. The various possible combinations of parental genotypes, together with the F1 genotypes and phenotypes expected in each situation, assuming that the genes are not linked and follow normal Mendelian inheritance, are presented in Table 3.4. For simplification, the recessive allelles defective in A/J and AKR are referred to as 'x' and 'y' respectively. and the corresponding dominant allelles conferring IL-3-responsiveness are referred to as 'x+' and 'y+'. If the parents are homozygous for both allelles (Table 3.4A), it would be expected that all progeny would be heterozygous for both allelles and would be responsive to IL-3. However, if the A/J parent is heterozygous for y, or the AKR parent is heterozygous for x, only 50% of the offspring would be expected to be heterozygous for both allelles and therefore responsive to IL-3 (Table 3.4B). Similarly, if both parents are heterozygous for one allelle, 25% of the F1 progeny would be expected to be responsive to IL-3 (Table 3.4C).

Bone marrow cells from the (AKR x A/J) F_1 did not produce colonies in response to IL-3 (Table 3.3). Similarly a further cross between A/J and AKR mice yielded non-responsive progeny (data not presented). Therefore, it is likely that the same gene or genes are defective in AKR and A/J mice. However, it is also possible that responsive phenotypes are produced from the A/J x AKR cross but were not detected due to the small samples of F1 progeny

sampled, or that the doubly heterozygous genotype which gives the responsive phenotype is fatal (or otherwise selected against) and was therefore not detected.

Table 3.3 Possible outcomes of A/J x AKR cross assuming that A/J and AKR are defective at different, unlinked loci

A. Parental Genotypes	(A/J) $x\ y^+$ $x\ y^+$		(AKR) $x^+\ y$ $x^+\ y$	
F1 Genotypes	$x\ y^+$ $x^+\ y$			
F1 Phenotypes (% total)	R (100%)			
B. Parental Genotypes	(A/J) $x\ y+$ $x\ y$		(AKR) $x^+\ y$ $x^+\ y$	
F1 Genotypes	$x\ y+$ x^+y	$x\ y$ x^+y		
F1 Phenotypes	R (50%)	N (50%)		
C. Parental Genotypes	(A/J) $x\ y+$ $x\ y$		(AKR) $x+\ y$ $x\ y$	
F1 Genotypes	$x\ y+$ $x\ y$	$x\ y$ $x\ y$	$x\ y$ $x+y$	$x\ y+$ $x+y$
F1 Phenotypes	N (25%)	N (25%)	N (25%)	R (25%)

R, responsive to IL-3. N, non-responsive to IL-3.

DISCUSSION

The analysis of the strain-dependent variation in the response of mouse bone marrow cells to various growth factors revealed that A/J and AKR mice are very poor responders to IL-3 (Chapter 2). However, although bone marrow cells from these 2 strains do not produce colonies with IL-3 in soft agar cultures, IL-3 does induce a low level of proliferation in liquid cultures of these cells. In the present Chapter, 5 alternative hypotheses were proposed which would account for these observations and explain the disparity between the soft agar and liquid culture assays. The results and conclusions from experiments designed to investigate each of these hypotheses are summarised below:

1. *In vitro* culture with IL-3 failed to expand an IL-3-responsive cell population from A/J bone marrow cells — a method which dramatically expands such a population from DBA/2 or BALB/c bone marrow cells. Therefore it seems unlikely that there are any IL-3-responsive CFCs in A/J bone marrow.

2. AKR bone marrow cells proved unresponsive to autologous IL-3. Furthermore, the activities of AKR IL-3 and rIL-3 on DBA/2 and AKR bone marrow cells were comparable indicating that there is no biologically significant difference between AKR IL-3 and BALB/c derived rIL-3.

3. A/J bone marrow cells bound [^{125}I]-IL-3 specifically, indicating that these cells do possess receptors for IL-3. The binding data suggests that IL-3 receptors may be present in lower numbers in

A/J bone marrow than in BALB/c, C57Bl or DBA/2 bone marrow. A Scatchard analysis would provide more conclusive data on IL-3 receptor numbers on bone marrow cells from each strain and would allow a comparison of the affinities of these receptors. However, further binding analyses were not performed due to limited availability of radioiodinated and biologically active IL-3.

4. The proliferation of A/J bone marrow cells in IL-3 was cell density dependent indicating that this response may not be directly induced by IL-3. Further analysis revealed a low level of non-IL-3 colony-stimulating-activity in media conditioned by A/J cells cultured in IL-3. Therefore, the proliferation observed in liquid cultures of A/J cells probably reflects a response to endogenous growth factors induced by IL-3 .

5. A/J cells cultured in IL-3 produce an activity which partially inhibits the IL-3 induced proliferation of cells from a normal IL-3 responder strain. This inhibitory activity may be prostaglandin E (PGE) which is induced by IL-3 in a number of macrophage and macrophage-like populations (Kurland et al.1977)and inhibits IL-3 induced proliferation (Kurland and Moore 1977; Moore,1982). Because the levels of inhibitory activity found in A/J conditioned media are not significantly greater than those found in media conditioned by DBA/2 cells, the defect in A/J mice is probably not caused by excessive endogenous inhibitor production. However, the data does not eliminate the possibility that the defect in A/J mice is caused by excessive sensitivity to the endogenous inhibitor.

Finally, the results from F_1 experiments suggest that the same abherrant gene or genes result in the poor IL-3 response of A/J and AKR mice and, as a corollary, the same fundamental defect is involved. This would justify the somewhat interchangeable use of the two strains which was necessary due to unpredictable supplies of these mice during the early work reported in this thesis. However further analyses, using larger samples of F1 mice, are required to thoroughly investigate the genetics of the defective A/J and AKR response.

The results presented in this Chapter lead to the conclusion that IL-3 has no direct mitogenic activity on A/J cells although it is not clear why this is so. Perhaps a subpopulation of stem cells from which IL-3-responsive CFCs derive is absent in the A/J strain. Alternatively, there may be a defect in the IL-3 receptor in A/J mice. It seems unlikely that the IL-3 receptor is completely defective or absent in A/J bone marrow, since there is an indirect response to IL-3 in cultures of A/J cells. However, the defect may lie in abnormal function of the IL-3 receptor or in reduced generation of second messengers. Indeed, such a defect has been proposed as the major cause of the reduced response of A/J macrophages to interferon-γ. Specifically, Hamilton et al. (1986) found that the binding of interferon-γ to its receptor on A/J macrophages did not induce either enhanced protein kinase C activity or elevation of intracellular calcium — biochemical changes previously shown to be associated with macrophage priming induced by interferon-γ. Therefore, it is tempting to speculate that perhaps the deficiency in the response of A/J bone

marrow cells lies in a signal transduction pathway in common with that of interferon-γ

In the light of the assumed importance of IL-3 in haemopoiesis, it seems suprising that the haemopoietic systems of AKR and A/J mice are apparently normal. There is mounting evidence however, that GM-CSF, initially considered to be specific for the proliferation of granulocyte/macrophage progenitors, also exhibits many of the activities attributed to IL-3. Purified recombinant human GM-CSF supports the formation of various types of single and multilineage colonies and augments erythroid colony formation, suggesting that the targets of GM-CSF include multipotential progenitors (Emerson et al.,1985, Sieff et al.,1985, Donahue et al.,1985). Furthermore, at high concentrations recombinant mouse GM-CSF appears to support the production of eosinophil, megakaryocyte and mixed-erythroid colonies (Metcalf et al.,1986b). Most recently, Koike and associates (1987) reported that GM-CSF supports multipotential colony formation from spleen cells of 5-FU treated mice. Therefore, it is conceivable that an adequate complement of haemopoietic cells can be produced via a predominantly GM-CSF controlled pathway. An alternative viewpoint, supported by the results presented in the following Chapter, is that although the direct proliferative activity of IL-3 is somewhat redundant, its synergistic activities with other factors such as M-CSF may be essential for the development of the haemopoietic system.

Chapter 4

Synergistic effect of IL-3 plus M-CSF on A/J bone marrow cells

INTRODUCTION

M-CSF stimulates the production of haemopoietic colonies which consist almost exclusively of macrophages, suggesting that the action of M-CSF is restricted to relatively late progenitors already committed to the macrophage lineage. However, early haemopoietic progenitors respond synergistically to M-CSF in combination with other haemopoietic factors.

Bradley and Hodgson (1979) originally described a population of CFCs, termed high-proliferative-potential CFCs (HPP-CFCs), which produce large colonies (> 0.5mm) in the presence of a source of M-CSF and a synergistic factor (SF). These HPP-CFCs are found in normal bone marrow and are enriched in bone marrow harvested from mouse donors four days post 5-FU treatment, suggesting that this cell population comprises relatively primitive haemopoietic precursors (as discussed in Chapter 1). Bradley and Hodgson found a number of sources of SF including human spleen, human placental and mouse spleen conditioned media (CM). Later, burst promoting activity (BPA), partially purified from WEHI-3B CM, was also shown to exhibit SF activity (Iscove et al.,1982). In retrospect, the SF present in WEHI-3 CM (and probably also in mouse spleen cell CM) is clearly IL-3 (McNiece et al.,1984), whereas the SF detected in the human CMs is similar if not identical to IL-1 (previously termed haemopoietin-1: Mochizuki et al.,1987; Zsebo et al.,1988).

Using clonal replating techniques, McNiece et al. (1986) demonstrated the existence of two populations of HPP-CFC which they termed HPP-CFC 1 and HPP-CFC 2. The HPP-CFC 1 population

is stimulated by the combination of M-CSF plus IL-1 and probably represents the most primitive haemopoietic cell population yet shown to proliferate and differentiate in culture. The HPP-CFC 2 population is actively generated by HPP-CFC 1 (McNiece et al.,1987), is stimulated by M-CSF plus IL-3 and generates macrophage-CFC (M-CFC) that differentiate to form mature macrophages.

In a model designed to explain the synergistic activity of IL-3, Iscove (Iscove et al.,1982) proposed that IL-3 is a lineage indifferent factor needed to expand a population of pluripotent haemopoietic precursors and their early comitted progeny whereas lineage specific factors such as M-CSF act only on later cells which have acquired the appropriate receptors as part of their differentiation programme. To delineate further the roles of IL-3 and M-CSF in macrophage colony formation in culture, Koike and associates (1986) investigated what effect delaying the addition of one factor had on the large colony response to IL-3 plus M-CSF. In soft agar bone marrow cell cultures where IL-3 was present from the initiation of the culture, the sizes of colonies produced were similar regardless of whether the addition of M-CSF was delayed for 5 days or not. However, when M-CSF was present from the initiation of the culture but the addition of IL-3 was delayed, only small colonies resulted. Consistent with the model proposed by Iscove (Iscove et al.,1982), these results suggest that M-CSF promotes cell proliferation during later stages of macrophage colony development that has been initiated by IL-3.

As a corollary of Iscove's model, the synergistic activity of IL-3 is dependent on its proliferative activity on early haemopoietic precursors. In other words, the synergistic response of bone marrow cells to IL-3 and M-CSF should follow the same strain-dependent profile as the response to IL-3 alone. To test this proposal, the synergistic large colony response to IL-3 and M-CSF was assayed in a range of strains including the IL-3 non-responders, A/J and AKR (Chapter 3). The results of these experiments are described in the following section along with further work to ascertain the contribution of the direct mitogenic activity of IL-3 in the synergistic response to IL-3 and M-CSF.

MATERIALS AND METHODS

CHEMICALS AND GROWTH FACTORS

Chemicals and growth factors used were as described in Chapter 2 unless otherwise stated.

METHODS

4.1 Assay for synergistic colony response

Colony assays were performed as described in method 2.3. The growth factors added were IL-3 (200U/ml), M-CSF (200U/ml), or IL-3 plus M-CSF. Control assays contained no added growth factors. Colonies > 0.5mm diameter were scored as large colonies. For identification of colony types, gels were fixed and stained as follows. Gels were placed on microscope slides, fixed in methanol for 30 seconds, rinsed with distilled water and air dried. Gels were then stained with α-naphthyl acetate esterase and naphthol AS-D chloroacetate (using Sigma kit) to allow identification of granulocytes and macrophages.

4.2 Bone marrow cell preculture

Precultures were performed as described in method 3.1 except that additional cultures containing M-CSF (200U/ml) were set up in parallel.

4.3 Sequential addition colony assays

Colony assays were performed as described in method 2.3 with the following modifications. Colony assays initiated (day 1) in the presence of IL-3 (200U/ml) were overlaid 2 days later (day 3) with either M-CSF (to give a final concentration of 200U/ml) or an equivalent volume of RPMI. Similarly, assays initiated in the presence of M-CSF (200U/ml) were overlaid 2 days later with either IL-3 (final concentration 200U/ml) or an equivalent volume of RPMI. Control assays without added growth factor or containing IL-3 plus M-CSF throughout the culture period were set up in parallel. Assays were incubated for 12 days before fixing and staining as in method 4.1. Colonies > 0.5mm diameter were scored as large colonies.

RESULTS

Synergistic effect of M-CSF and IL-3

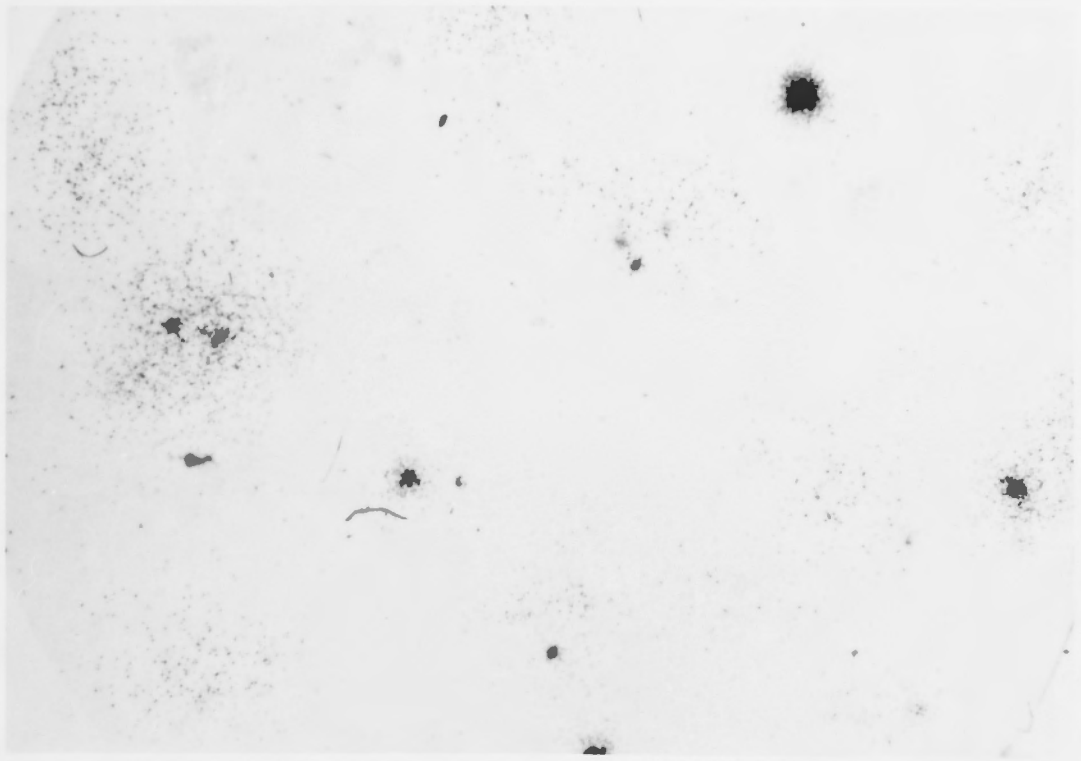
To determine whether or not the synergistic response follows the same strain-dependent pattern as the response to IL-3 alone, the combination of IL-3 and M-CSF was tested in colony assays (method 4.1) on the same panel of mouse strains used in Chapter 2. In soft agar cultures of bone marrow cells (isolated using method 2.1) from all strains tested including AKR and A/J, the presence of IL-3 plus M-CSF resulted in much larger colonies than were produced in M-CSF or IL-3 alone (Figs 4.1 and 4.2). In some assays a few large colonies (> 0.5 mm diameter) were produced in M-CSF alone, but these never exceeded 10% of the total colony number. In comparison, in IL-3 plus M-CSF the percentage of large colonies ranged from 25% to 40% of the total colony number. In assays using bone marrow cells from IL-3 responsive strains, the number of colonies supported by the combination of IL-3 and M-CSF was less than or equal to the sum of the colonies supported by the factors individually (colony scores from DBA/2 bone marrow cells are presented in Table 4.1). In A/J and AKR assays however, the synergistic response to IL-3 plus M-CSF was also reflected in a significant increase in colony numbers (Table 4.1).

We next examined the effect of IL-3 on the type of colonies produced from A/J bone marrow cells in soft agar cultures. At high concentration, M-CSF alone supported exclusively macrophage colonies, whereas at lower concentrations some granulocyte (G) and granulocyte/macrophage (GM) colonies were produced. The

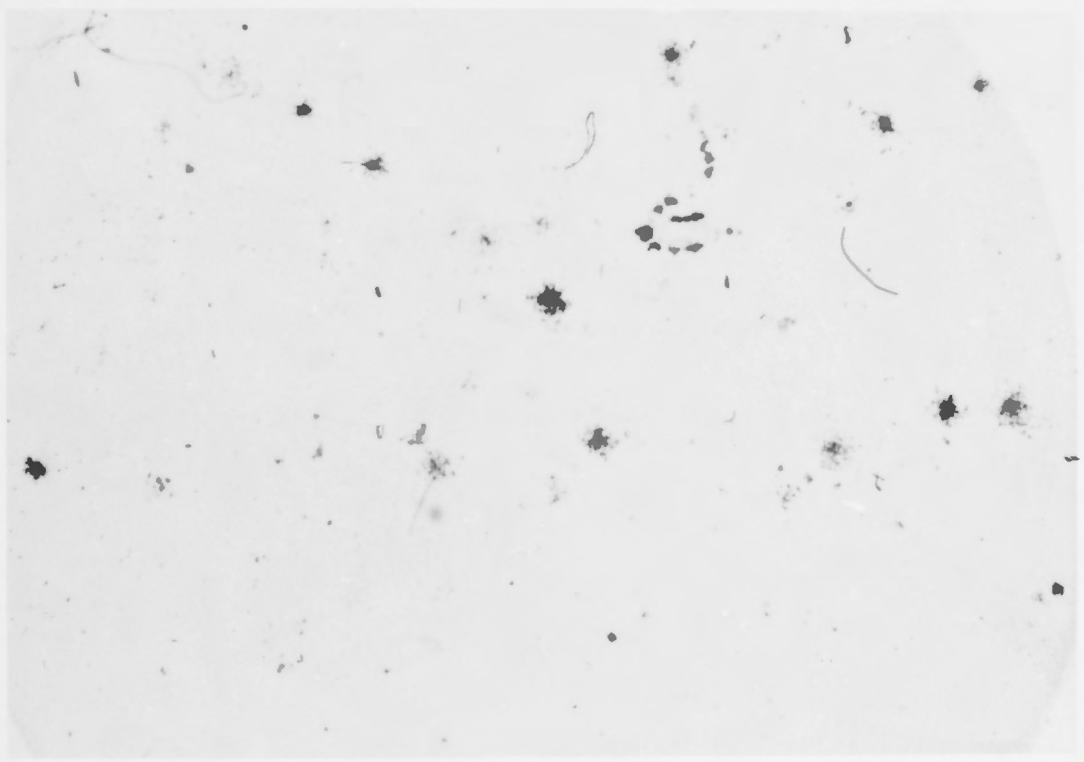
Fig 4.1 Synergistic effect of IL-3 plus M-CSF on the size of colonies produced from DBA/2 bone marrow cells

Colonies grown from 1 x 10^4 DBA/2 bone marrow cells with (A) IL-3, (B) M-CSF or (C) IL-3 + M-CSF. Photographs are of whole agar preparation (16 mm diameter) stained with α-naphthyl acetate esterase and naphthol AS-D.

A.



B.



C.

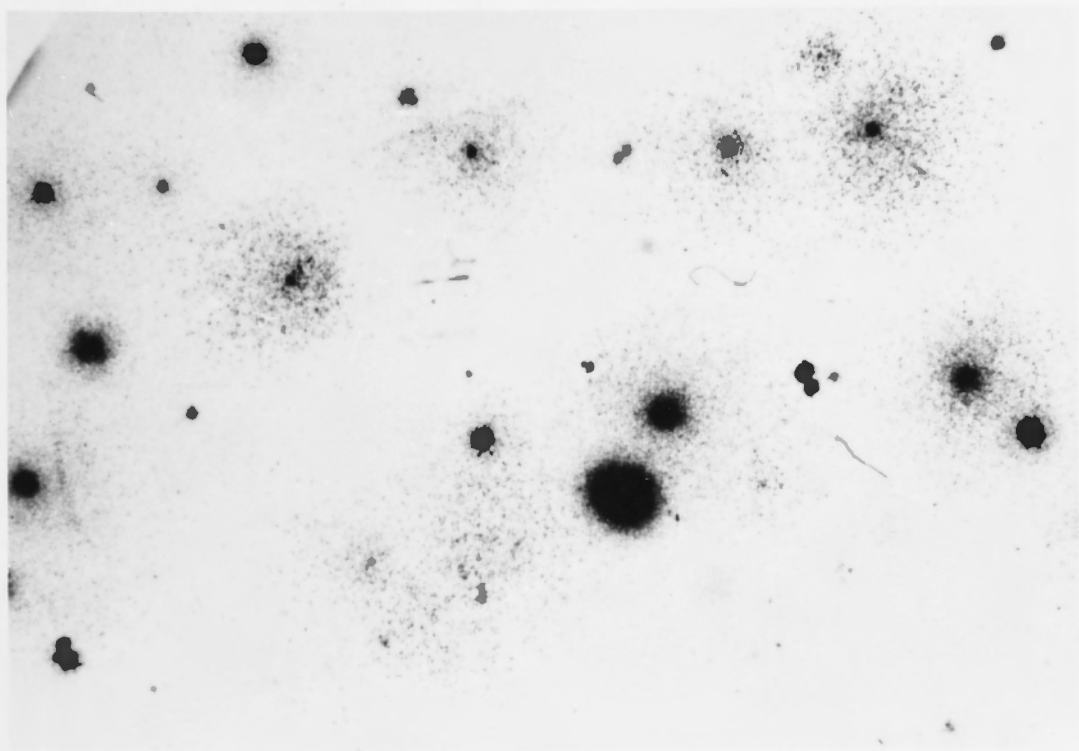
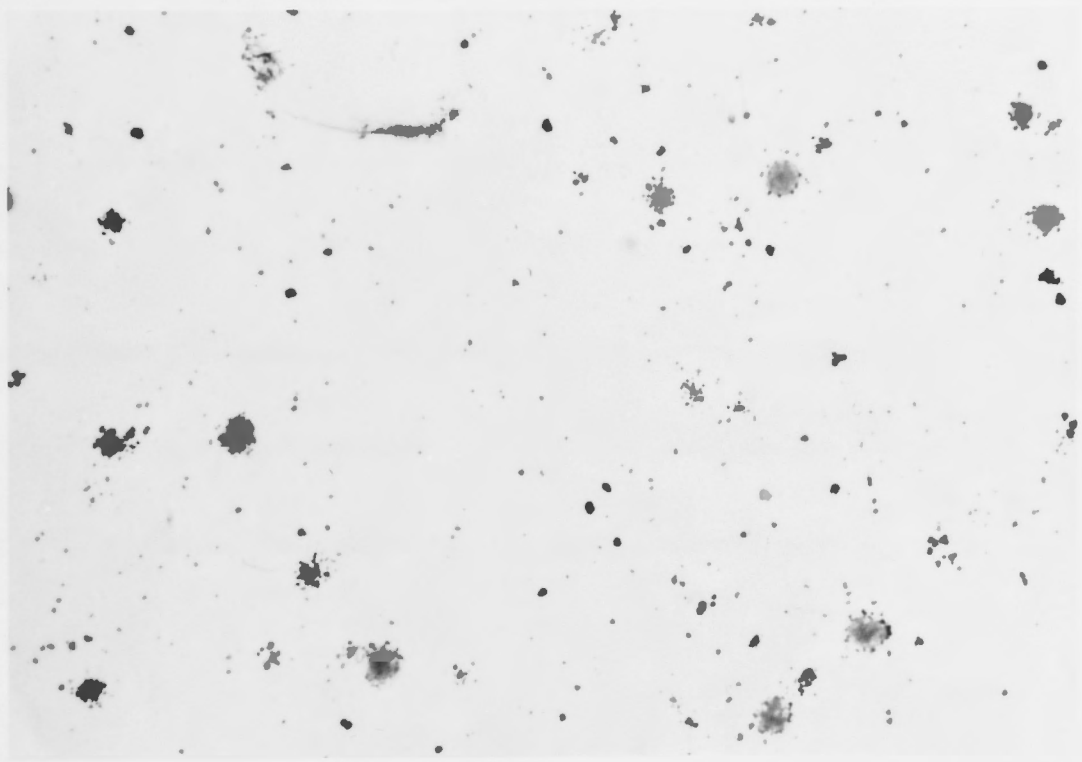


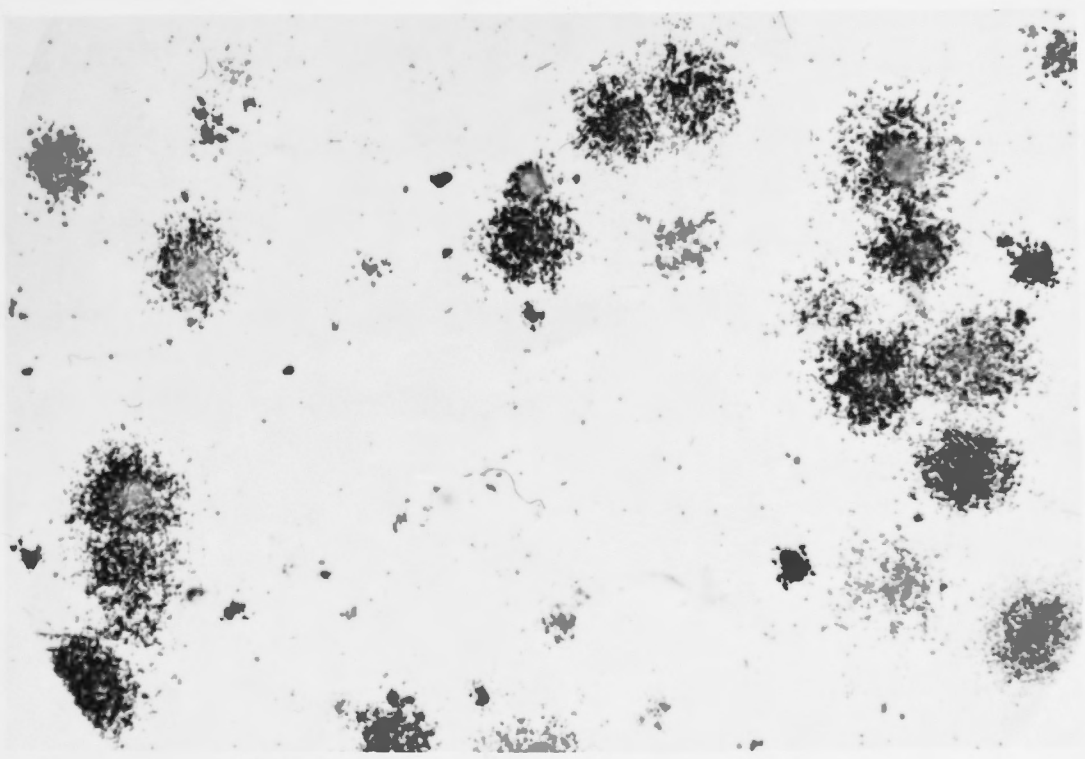
Fig 4.2 Synergistic effect of IL-3 plus M-CSF on the size of colonies produced from A/J bone marrow cells

Colonies grown from 1 x 10^4 A/J bone marrow cells with (A) M-CSF or (B) IL-3 + M-CSF. No colonies were produced in response to IL-3 alone. Photographs are of whole agar preparation (16 mm diameter) stained with α-naphthyl acetate esterase and naphthol AS-D

A.



B.



presence of IL-3 increased the percentage of G and GM colonies produced at all M-CSF concentrations (Table 4.2).

Table 4.1. Colony formation from bone marrow cells stimulated by IL-3 and/or M-CSF

	Number of Colonies					
Factor	A/J		AKR		DBA/2	
IL-3	0	0	0	0	44±3	48±3
M-CSF	25±3	10±1	14±5	15±2	22±2	19±2
IL-3 + M-CSF	34±3	22±5	27±4	24±1	52±4	53±3

Data represents the mean ± SD or the number of colonies in triplicate cultures each containing 1 x 10^4 cells. Two independent experiments on each strain are tabulated. The numbers of colonies produced from A/J and AKR bone marrow cells plated in IL-3 + M-CSF are significantly different from the number produced in M-CSF alone ($P < 0.01$) according to the Students t test.

Table 4.2 Types of colony produced by A/J bone marrow cells stimulated with M-CSF in the presence or absence of IL-3

Factors	Colony number	Colony type (%)		
		M	G	GM
100U M-CSF	10 ± 4	84	13	3
100U M-CSF + IL-3	12 ± 1	75	14	11
200U M-CSF	10 ± 1	93	7	0
200U M-CSF + IL-3	22 ± 2	77	17	6
400U M-CSF	12 ± 2	100	0	0
400U M-CSF + IL-3	23 ± 3	92	4	4

M-CSF concentrations shown are the final concentration in the culture (U/ml), final concentration of IL-3 was 200U/ml in all assays. Colony numbers are the mean ± SD of the colony counts from triplicate cultures each containing 1 x 10^4 cells. M, macrophage; G, granulocyte; GM, granulocyte-macrophage

Effect of M-CSF on response of A/J bone marrow cells to IL-3

The increased number and variety of colonies are produced from A/J bone marrow cells in IL-3 plus M-CSF compared with M-CSF alone, suggested that exposure to M-CSF primes a population of A/J CFCs to become IL-3 responsive. To investigate this possibility A/J bone marrow cells were precultured in M-CSF (method 4.2), and the non-adherent cells were then harvested. The yield of non-

adherent cells was not significantly different from the original number of bone marrow cells plated but large numbers of adherent cells (not harvested for colony assays) were also observed in all these cultures. Even after this pre-exposure to M-CSF, A/J bone marrow cells did not produce colonies when cultured in soft agar (method 2.3) in the presence of IL-3 alone (Table 4.3a). Subsequent experiments in which A/J bone marrow cells were precultured with M-CSF for various lengths of time (1 day, 4 days, and 6 days) also failed to produce IL-3 responsive CFCs (data not presented).

Effect of IL-3 on A/J bone marrow cell response to M-CSF

Preculture experiments (method 4.2) were also performed to determine whether exposure to IL-3 affected the response of A/J bone marrow cells to M-CSF. As expected, IL-3 preculture did not result in a significant increase in overall numbers of non-adherent A/J bone marrow cells. We plated precultured and fresh bone marrow cells in soft agar culture with M-CSF (method 2.3) and scored the number and size of the colonies produced 7 days later. The number of large colonies produced by the precultured cell population was not significantly greater than the number produced by fresh bone marrow (Table 4.3b) indicating that IL-3 precultured cells were not preactivated to produce large colonies in response to M-CSF. In addition IL-3 preculture did not expand the population of M-CSF-responsive CFCs compared with fresh bone marrow. However, A/J bone marrow precultured with IL-3 contained more non-adherent M-CSF responsive CFCs than parallel cultures with M-CSF or without added growth factor (Table 4.3a,b).This experiment was repeated twice with similar results (data not represented).

Table 4.3. Colony formation by A/J bone marrow cells precultured with M-CSF or IL-3

	Number of Colonies		
Factor in Preculture	IL-3	M-CSF	IL-3+M-CSF
(a) M-CSF	0	8±4 (0%)	17±3 (12%)
(b) IL-3	0	24±4 (0%)	32±3 (16%)
No factor	0	4±1 (0%)	9±1 (14%)
Control (fresh bm cells)	0	16±4 (0%)	33±8 (27%)

Colony numbers are the mean ± SD of the colony counts from triplicate cultures each containing 1 x 10^4cells. Numbers in parentheses represent the large (> 0.5 mm diameter) colony count expressed as a percentage of the total colony counts. Bm, bone marrow.

Effect of sequential additions of CSFs on colony size and number

Finally, experiments were carried out to determine what effect delaying the addition of one of the growth factors would have on the IL-3 plus M-CSF induced synergistic large colony response by A/J bone marrow cells. Bone marrow cells from A/J mice were plated in colony assays (method 4.3) in the presence of either IL-3 or M-CSF at the beginning of the culture period (day 1). On day 3, the second factor was added. Control assays contained IL-3 plus M-CSF from day 1 of the culture. The results of these experiments were variable but the overall pattern was consistent in that delaying the addition of either factor reduced the percentage of large colonies produced (Table 4.4) but had no effect on the overall number of colonies produced.

Table 4.4. Effect of sequential addition of IL-3 and M-CSF on colony size

Growth Factor Added		%LARGE COLONIES		
DAY 1	DAY 3			
IL-3 + M-CSF	-	30	15	26
IL-3	M-CSF	9	4	18
M-CSF	IL-3	16	12	24
M-CSF	-	9	0	1

Results of 3 independent experiments are tabulated. Large colonies are > 0.5 mm diameter.

DISCUSSION

Previous reports have documented that a population of haemopoietic progenitor cells produces distinctively large colonies in the presence of IL-3 plus M-CSF (Iscove et al.,1982; McNiece et al.,1984; Koike et al.,1986). The effect of this combination of growth factors on bone marrow cells from a range of mouse strains is presented here. As expected, bone marrow cells from IL-3-responsive strains produced larger colonies in IL-3 plus M-CSF than in either factor alone. In these assays the number of colonies produced in the combination of IL-3 plus M-CSF was no more than additive. Similarly, Williams and associates (1987a) reported that the synergism between IL-3 and M-CSF at optimal concentrations, enhanced the size but not the number of colonies produced from granulocyte/macrophage progenitors. In contrast, in A/J bone marrow cell cultures, IL-3 and M-CSF acted synergistically to increase both the size and the number of colonies produced compared with those produced in M-CSF alone. Furthermore, A/J bone marrow cells cultured in IL-3 plus M-CSF generated G, GM, and M colonies whereas in M-CSF alone only M colonies were found.

A hypothesis consistent with these observations is that M-CSF is required to prime haemopoietic progenitor cells to respond to IL-3. As a corollary, in IL-3 responsive strains this priming event must occur before the cells are isolated for *in vitro* experimentation. The experimental evidence presented here does not support this hypothesis however, since exposure to M-CSF does not preactivate A/J cells to produce colonies in response to IL-3. This does not rule out the possibility that continuous exposure to low levels of

endogenously produced M-CSF is required to prime an IL-3 responsive population and that this endogenous production of M-CSF is lacking in A/J mice. This too is unlikely however, firstly because single bone marrow cells isolated from CBA mice proliferate in response to IL-3, indicating that the action of IL-3 on these cells does not require mediation by a second factor released by another cell type (Clark Lewis and Schrader, 1988) and secondly because there is no apparent difference in the endogenous growth factor production by A/J bone marrow cells and bone marrow cells from the IL-3 responder strain DBA/2 (Chapter 3).

An alternative hypothesis to explain the increased number of colonies in IL-3 plus M-CSF is that IL-3 primes an A/J haemopoietic stem cell population to respond to M-CSF. In support of this hypothesis, Bartelmez and associates demonstrated that in C3H/HeJ bone marrow cell cultures, IL-3 stimulates the *de novo* appearance of blast cells possessing the M-CSF receptor (Bartelmez et al.,1985). It is not clear from the present study whether the observed synergistic increase in colony numbers produced from A/J bone marrow cells in IL-3 plus M-CSF is due to stimulation of the *de novo* appearance of M-CSF responsive CFCs by IL-3. No significant increase in the number of M-CSF responsive CFCs in A/J bone marrow was found after IL-3 preculture but this effect may be obscured by the differentiation and subsequent adherance of a proportion of the macrophage progenitors during culture. In favour of this interpretation, a synergistic increase in colony numbers was observed in colony assays in which IL-3 was present from the initiation of the culture but the addition of M-CSF

was delayed, whereas a corresponding increase in IL-3 plus M-CSF responsive CFCs was not observed after IL-3 preculture.

Another possible explanation for the observed synergistic increase in colony numbers however, is that coincident exposure to IL-3 and M-CSF is required to prime a population of A/J CFCs to proliferate. The synergism between IL-1 and M-CSF is apparently an example of such an interaction: Using a highly purified multipotent stem cell population, Lord and Spooncer (1986) demonstrated that coincident exposure to IL-1 and M-CSF is required to commit these early cells to an M-CSF-responsive state. However, further work with purified early progenitor populations from the A/J strain is required to clarify whether there is a population of cells requiring coincident exposure to IL-3 and M-CSF for proliferation.

Apart from increasing the numbers of colonies produced from A/J bone marrow cells, the presence of IL-3 and M-CSF increased the size and variety of colonies produced compared with those produced in M-CSF alone. The increase in colony size appears to require coincident exposure to both IL-3 and M-CSF since exposure to IL-3 did not preactivate a population of A/J haemopoietic progenitor cells to produce large colonies when plated subsequently in M-CSF alone. The increase in variety of colony types may be accounted for partly by the stimulation of earlier less committed progenitor cells by the combination of growth factors. However, since M-CSF has a strong differentiative activity - such that exposure of bipotential GM-CFC to M-CSF through 2 or 3 cell divisions irreversibly commits the cells to form macrophage

colonies (Metcalf and Burgess 1982) - it appears that IL-3 must also antagonise this M-CSF driven differentiation.

The sequential additions experiments reported here revealed that both IL-3 and M-CSF must be present from the initiation of the culture to achieve the optimum large colony response from A/J bone marrow cells. In contrast, Koike et al. (1986) found that delaying the addition of M-CSF had no effect on the large colony response of bone marrow cells from the IL-3-responsive strain BDF1. Taken together, these observations suggest that the production of synergistic large colonies requires both a synergistic activity and a proliferative stimulus. Since bone marrow cells from BDF_1 mice produce relatively large colonies in culture with IL-3, both the synergistic activity and the proliferative stimulus can be provided by IL-3 in the early stages of large colony development from these bone marrow cells. In the A/J cultures however, IL-3 provides the synergistic activity but M-CSF is required for the proliferative response throughout the culture period.

In conclusion, the data presented in this Chapter indicate that IL-3 and M-CSF synergise on A/J bone marrow cells in the absence of direct IL-3-induced proliferation. This is obviously inconsistent with Iscove's model which proposes that the synergistic activity of IL-3 is entirely due to its proliferative action on early haemopoietic progenitor populations. However, the separation of the synergistic from the mitogenic effects of IL-3 in the A/J system provides an ideal model for studying what mechanisms <u>are</u> actually involved in the synergistic activities of IL-3.

Chapter 5

Effect of IL-3 on expression of c-*fms* transcripts by A/J bone marrow cells

INTRODUCTION

As discussed in Chapter 4, in soft agar cultures of haemopoietic progenitor cells, IL-3 acts synergistically with M-CSF to produce very large colonies. The mechanism previously suggested for this synergism is that IL-3 initiates the colonies by amplifying immature early macrophage progenitors which subsequently develop sensitivity to M-CSF (Koike et al.,1986). However, since IL-3 synergises with M-CSF even in the A/J system, in which IL-3 has no direct proliferative activity (Chapter 4), the proliferative activity of IL-3 is apparently not essential to its role in enhancing the response to M-CSF.

The development of M-CSF sensitivity by haemopoietic progenitor cells has been correlated to an increase in M-CSF receptor expression (Bartelmez et al.,1985, Bicknell et al.,1988). The mouse M-CSF receptor is a 165 kd glycoprotein which has tyrosine-specific protein kinase activity (Morgan and Stanley,1984; Yeung et al.,1987) and is similar in its properties to the product of a feline proto-oncogene c-*fms* (Rettenmier et al.,1985). Indeed a rabbit antiserum prepared using a transmembrane glycoprotein encoded by the viral oncogene (v-*fms*) has been shown to have specific cross-reaction with the mouse M-CSF receptor (Sherr et al.,1985). Moreover, v-*fms* transformed fibroblasts express high affinity binding sites for M-CSF (Sacca et al.,1986). These results implicate the M-CSF receptor as a similar if not identical molecule to the product of the c-*fms* proto-oncogene.

The aim of the experiments reported in this chapter was to investigate whether the effect of IL-3 on M-CSF-responsiveness could be linked with an increase in c-*fms* RNA expression.

MATERIALS AND METHODS

CHEMICALS, GROWTH FACTORS AND PLASMIDS

The chemicals and growth factors used were as described in Chapter 3 unless otherwise stated. The 0.4 kb *Pst*1 fragment of v-*fms* cDNA inserted into pBR322 was kindly donated by T.Robbins (NCI-FCRF, Maryland, USA)). The plasmid pUB8 which contains 2.8 human ubiquitin coding units inserted into the plasmid pUC18 (Baker and Board,1987) was kindly donated by R.Baker, Department of Human Genetics, JCSMR, ANU, Canberra)

METHODS

5.1 Culture of freshly isolated bone marrow cells and bone marrow-derived macrophages

Bone marrow cells (isolated using method 2.1) were cultured at 1 x 10^6/ml in 75 ml RPMI-10% FCS in the presence of IL-3 (200U/ml), M-CSF (200U/ml), IL-3 plus M-CSF in 75 cm^2 plastic tissue culture flasks (Falcon Labware). Cultures were incubated at 37^oC in a humidified 5% CO_2 atmosphere. After the time specified in the text, cells were harvested and lysed as described in method 5.4.

5.2 Production of bone marrow derived macrophages

Bone marrow-derived macrophages were produced from liquid cultures of mouse bone marrow cells in the presence of L-cell conditioned medium (LCM) as described by Hume and Gordon (1983).

Briefly, bone marrow cells were harvested from mouse femurs (method 2.1) and cultivated in a medium containing 20% LCM. After 5 days, the non-adherent cells were discarded and the adherent cells were washed *in situ* with RPMI-10% FCS before use.

5.3 Maintenance of cell lines

Mouse L929 cells, which constitutively produce M-CSF, were grown in DMEM (Gibco) plus 5% FCS. Cells were harvested with trypsin (1.25% w/v)-EDTA (1.5 mM) in PBS every 7 days and replated at one-tenth density. WEHI-3B cells, which constitutively produce IL-3, were grown in RPMI-5% FCS. Non-adherent and adherent cells were harvested by agitation and replated at one-tenth density every 7 days. The mouse cell line PU5 which constitutively expresses GM-CSF RNA was cultured as described for L929 cells.

5.4 Isolation of total RNA by ultracentrifugation

This method is based on that of Muller et al. (1983). To avoid contaminating the samples with RNase, gloves were worn throughout the procedure. Solutions and buffers which did not contain RNase inhibitors were incubated with diethyl pyrocarbonate (DEPC) at room temperature for at least 12 h and autoclaved (Maniatis et al.,1982). Sterile disposable plasticware was used for the preparation and storage of RNA without pretreatment. General laboratory glassware was treated by baking at 250°C for at least 4 h.

Cultured cells or cells prepared by tissue disaggregation, were pelleted by centrifugation and resuspended in a minimum volume of PBS and the resulting cell suspension was added dropwise into lysis buffer with vigorous vortexing. Approximately 10 ml of lysis buffer was used per 10^8 cells or gram of tissue. Cells grown in monolayer or adherent cell populations from bone marrow cell cultures were lysed directly in the tissue culture dish or flask by addition of lysis buffer (4 ml of lysis buffer for 75 cm^2 flask). The lysis buffer contained 4 M guanidine thiocyanate, 0.4% lauroyl sarcosine, 0.025 M sodium citrate (pH7.0), 0.1% Antifoam A and0.1 M 2-mercaptoethanol (added just before use; BDH Chemicals Ltd.) Tissue or cell lysate was then forced through a 21 guage needle several times to shear the DNA and either processed immediately, following the protocol below, or stored at -70^oC until required.

The lysate, made up to 30 ml with lysis buffer, was underlayered with 5 ml of 5.7 M caesium chloride in 0.025 M sodium acetate (pH 5.0) in an SW27 polyallomer tube (Beckmann) and centrifuged at 23 K rpm at 15^oC for 24 h in a Beckmann SW27 rotor. After carefully withdrawing the guanidine isothiocyanate layer, the caesium chloride cushion was decanted and the tubes were left to drain for about 10 min. Each tube was then cut below the interphase level and the bottom part of the tube containing the RNA pellet was rinsed with cold ethanol and allowed to drain. The RNA pellet was resuspended in 0.4 ml water. A 2.6 ml aliquot of 7.5 M guanidine hydrochloride buffer containing 0.025M sodium citrate (pH7.0) and 0.005 M dithiothreitol (DTT) was added to the RNA solution and the pH was lowered by addition of 75 µl of 1 M acetic acid. Cold ethanol (1.5 ml) was then added and the mixture was vortexed briefly and

stored at -20°C for at least 4 h. The resulting RNA precipitate was recovered by centrifugation at 10,000 x g for 15 min and the pellet was resuspended in 1.5 ml water and repeatedly drawn up and down a pasteur pipette until dissolved. The RNA was then reprecipitated by addition of 100 µl of 3M sodium acetate and 4 ml cold ethanol. After overnight precipitation at -20°C the RNA was recovered by centrifugation at 10,000 x g for 15 min and dissolved in 1 ml or other appropriate volume of water. The concentration of RNA was determined spectrophotometrically based on an optical density at 260 nm (OD_{260}) of 24 for 1 mg of RNA per ml. RNA was stored at -70°C until required. For long term storage (> 3 months) RNA was reprecipitated with ethanol, centrifuged and the pellet was stored under ethanol at -70°C.

5.5 Isolation of total RNA by single-step method

This method is essentially as described by Chomczynski and Sacchi (1987). Cells were lysed as in method 5.4. Lysate (3 ml) was placed in a 10 ml disposable plastic centrifuge tube. 0.3 ml of 2 M sodium acetate (pH 4), 3 ml of water saturated phenol (BDH Chemicals Ltd; prepared as described in Maniatis et al.,1982), and 0.6 ml of chloroform-isoamyl alcohol mixture (49:1) were added sequentially to the lysate with thorough mixing by inversion after the addition of each reagent. The final suspension was shaken vigorously for 10 seconds and cooled on ice for 15 min at 4°C. Samples were then centrifuged at 10,000 x g for 20 min at 4°C. The aqueous upper phase which contained RNA was transferred to a fresh tube and the interphase and phenol phase were discarded. The aqueous phase was mixed with 6 ml of ethanol by vortexing and then placed at -20°C

for at least 1 h to precipitate RNA. Samples were centrifuged at 10,000 x g for 20 min at 4°C to sediment the RNA precipitate. The resulting RNA pellet was then dissolved in 0.3 ml of lysis buffer, transferred to a 1.5 ml Eppendorf tube and reprecipitated with 0.6 ml of ethanol at -20°C for at least 1 h. After centrifugation in an Eppendorf centrifuge for 10 min at 4°C, the RNA pellet was resuspended in 75% ethanol, sedimented, vacuum dried (15 min) and dissolved in an appropriate volume (usually 50 or 100 ml) of water at 65°C for 10 min. Concentration of RNA was determined as in method 5.4.

5.6. Isolation of messenger RNA from total RNA

Poly (A)+ RNA (mRNA) was separated from total RNA by chromatography on oligo (dT)-cellulose (Aviv and Leder,1972). 0.5 - 1.0 g oligo (dT)-cellulose was equilibrated in loading buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.5 M NaCl) and poured into a sterile polypropylene column (Bio-rad) to give a final bed volume of 3 - 4 ml. Up to 3 mg of RNA was heated at 60°C for 5 min, rapidly chilled in ice water and adjusted to 0.5 M NaCl. The sample was then loaded onto the column and washed through with loading buffer until the OD_{260} of the column effluent was less than 0.005 (usually approx. 5 column volumes of buffer). At that point, the buffer was replaced with elution buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and 0.5 ml fractions were collected in Eppendorf tubes. Elution of mRNA was monitored by absorbance at 260 nm. The mRNA was ethanol precipitated overnight at -20°C, pelleted for 15 min in an Eppendorf centrifuge and resuspended in an appropriate volume of water.

5.7. Preparation of probe templates

The v-*fms* insert was excised from PBR322 using *Pst*1 and isolated by low melting point agarose gel electrophoresis and phenol extraction as described in method 5.8. The entire plasmid PUB8 was used as a template for the ubiquitin probes.

5.8 Isolation of DNA from low melting point agarose gels

Low gelling temperature agarose was dissolved by heating in electrophoresis buffer to 70°C. The agarose was allowed to cool to 37°C prior to pouring. Ethidium bromide was added to a final concentration of 0.5 µg/ml and the gel was poured in a cold room (4°C). Electrophoresis of DNA samples was carried out at 4°C at 100 mA constant current. Electrophoresis buffer was 40 mM Tris-acetate (pH 7.8), 1 mM EDTA and contained ethidium bromide at 0.5 µg/ml. Following electrophoresis, the gel was viewed under UV illumination and the region of the gel containing the fragment of interest was cut out using a scalpel blade. Approximately 0.5 ml gel slices were placed into separate Eppendorf tubes and incubated at 65°C for 5 min. At room temperature, 0.5 - 1 volume of phenol was added quickly to each tube and the tube was vortexed vigorously for 2 min. The phases were separated by centrifugation and the aqueous phase was then re-extracted twice with 1 volume of phenol then twice with 1 - 2 volumes of ether (water saturated). The resulting aqueous phase was heated for 2 min at 65°C to remove any remaining traces of ether and the DNA was recovered by ethanol precipitation.

5.9. Preparation of labelled DNA markers

A mixture of stock plasmid DNA was used which gave fragments of appropriate sizes when digested with *Eco*R1. Plasmid DNA (3 µg)was incubated with 3 units of *Eco*R1 in 45 ml restriction digest buffer (100 mM NaCl, 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT) at 37°C. After 1 h, 20 nmol dTTP, 3.3 pmol [α-^{32}P]-dATP (Amersham) and 5 units *Escherichia coli* DNA polymerase 1 (Klenow fragment, BRESA) were added to the digest in a total volume of 3 µl. The mixture was incubated at room temperature for 30 min and then made up to 300 µl with 100 mM Tris-HCl (pH 7.5), 1 mM EDTA. The resulting end labelled DNA fragments were stored at -20 C until they were used as size markers on Northern gels.

5.10 RNA slot blots

RNA slot blots were performed using a modification of the method of White and Bancroft (1982). RNA was dissolved in water to a concentration of 1 mg/ml. 15 µl of formaldehyde (37% w/w, Merck A.R.) and 30 µl of 20 x SSC (3 M NaCl, 0.3 M Na citrate) were added to 15 µl (15 µg) RNA in an Eppendorf tube. The mixture was vortexed, incubated at 60°C for 15 min and kept on ice. 60 µl of 20 x SSC was then added to each sample giving a final concentration of 15 x SSC. Serial three-fold dilutions of the denatured RNA were prepared in 15 x SSC to give final RNA concentrations of 10, 3.3, and 1µg / 80µl. RNA was applied to nitrocellulose (NC, Schleicher and Schuell) using a slot blot apparatus (Minifold 11, Schleicher and Schuell) as follows. 2 layers of Whatman 3MM paper were placed in the apparatus and a piece of NC, prewetted with 15 x SSC

was placed on top of the paper. The apparatus was assembled and 100 µl of 15 x SSC was pipetted into each of the sample wells to keep the NC wet until samples were loaded. The vacuum source was connected and 80 µl aliquots of RNA solution were pipetted into the appropriate wells. Once the samples had run through, each well was rinsed with 100 µl of 15 x SSC under vacuum. The vacuum source was then turned off and the minifold was dismantled, gently removing the NC filter with 'soft' forceps (Amicon Corp.). After air drying on 3MM paper, the filter was baked in a vacuum oven for 2 hr.

5.11 Northern blots

RNA was separated according to size on formaldehyde/agarose gels using a modification of the method described by Gurney (1984). A mini-gel apparatus (Pharmacia Biotechnology) kept free from RNase was used for all Northern gels. The gel buffer in the gel, in the sample and in the buffer reservoirs was: 20 mM triethanolamine (pH7.4), 2.5 mM EDTA, and 2.2 M formaldehyde. Sample preparation buffer was prepared just before use by mixing 10 volumes of deionised formamide (prepared as described in Maniatis et al.,1982) with 4 volumes of 5 x gel buffer. RNA solution (4 µl usually at 5 mg/ml) was added to 9 µl of sample preparation buffer in an Eppendorf tube and mixed thoroughly by vortexing the capped tube vigorously. The samples were then incubated at 55°C for 15 min and kept on ice. Labelled DNA size markers were denatured using identical conditions. Loading buffer (1 µl) containing 50% glycerol, 1 mM EDTA, 0.4% bromophenol blue and 0.4% xylene cyanol was added to each of the samples which were

then dry-loaded into wells of a 30 ml formaldehyde/agarose gel. The samples were run into the gel at 70 mA for 15 min and the gel was submerged with gel buffer. Electrophoresis was continued for 3 - 5 h at 35 - 45 mA (usually until the first dye front had migrated 50 - 70 % of the length of the gel). The gel was blotted to NC without pretreatment using established methods (Maniatis et al., 1982) with the following modifications. The transfer was left for 18 - 24 h at room temperature, after which the NC was removed and air dried on fresh 3MM filters. At this point the 2 major ribosomal (r) RNA bands could be detected by holding the NC up to the light and the positions of these bands were marked for future reference. The rRNA bands in each lane were compared to check that roughly equivalent amounts of each sample of RNA had been transferred.

5.12 Hybridisation of Northern and dot blots

A. Prehybridisation

Blots were prehybridized in 15 - 20 ml of 50% freshly deionised formamide, 5 x SSC, 0.1% sodium dodecyl sulphate (SDS), 5 x Denhardt's solution (Denhardt, 1966) and 200 µg/ml sonicated salmon sperm DNA (prepared as described in Maniatis et al.,1982) at 50°C for > 3 h. Prehybridisations and hybridisations were both carried out in small flat-bottomed plastic boxes with sealable lids in a gently shaking water bath.

B.Probe preparation by random primer synthesis

This method is based upon the procedure described by Feinberg and Vogelstein (1983, 1984) and can be used for labelling DNA from any source without the need for specific vectors or subcloning.

A typical probe synthesis reaction was as follows. 100 ng of the template DNA fragment or plasmid was mixed with a 20-fold (w/w) excess of a universal decamer primer (synthesized by the phosphoramidite method, using an equimolar mixture of all four bases at each position) in a volume of 10 µl. The mixture was sealed inside a drawn-out glass capillary and heated to 100°C for 5 min, followed by rapid chilling in ice water. The contents of the capillary were then added to an Eppendorf tube containing a final reaction mixture of 50 mm K phosphate (pH 6.8), 10 mM Mg acetate. 10 mM DTT, 1 mM each of dCTP, dGTP, dTTP (Boehringer Mannheim Biochemicals), 33 pmol [α - ^{32}P]dATP (Amersham) and 10 units of DNA polymerase (Klenow fragment) in 90µl. The reaction was incubated at 37°C for 60 min.

Probes were also prepared by the standard Multiprime DNA labelling protocol (Amersham). Using this protocol 50 ng of DNA was labelled with 10 µl (33 pmol) of [α - ^{32}P]dCTP (Amersham).

To monitor the progress of the reaction, the incorporation of label into trichloroacetic acid (TCA) precipitable counts was determined by the following method. A 1.5 µl aliquot was taken from the reaction mixture and added to 28.5 µl of 0.5 M EDTA (pH 8.0) in an Eppendorf tube. After vortexing the mixture, 10 µl was removed and

set aside for determination of the total radioactivity in the reaction mixture. Another 10 µl was added to 90 ml of 500 µg/ml calf thymus DNA in 10 mM Tris HCl (pH 7.5) and 10 mM EDTA. To this mixture, 2 ml of ice cold 10% TCA solution was added and the DNA was left to precipitate in an ice-bath for 10-15 min. Precipitated DNA was then collected by vacuum filtration on a glass fibre disc (Whatman). After washing the filter disc several times with cold 10% TCA solution and then with ethanol, the disc was placed in a scintillation vial with 10 ml ethanol and incorporated radioactivity was determined by Cherenkov counting. The sample set aside for determination of total radioactivity was counted under the same conditions as the filter disc. The % label incorporated could then be calculated.

The synthesized probe was separated from unincorporated label by chromatography on a Sephadex G-50 fine (Pharmacia Biotechnology) column (prepared in a 5 ml disposable pipette). The specific activity of probes produced was approx. 1-1.8 $x10^9$ dpm/µg

C. Hybridisation

Just prior to use, the probe was denatured in 15 - 20 ml of prehybridisation buffer at 70°C. Solution used for prehybridisation was discarded and replaced with prehybridisation buffer containing the denatured probe. Blots were hybridised overnight (18 h) at 50°C.

D. Washing

Filters were removed from hybridisation buffer and immediately submerged in a solution of 2 x SSC at room temperature. Filters were then incubated sequentially in 2 x SSC plus 0.1% SDS, 0.5 x SSC plus 0.1% SDS, 0.1 x SSC plus 0.1% SDS, for 30 min each, with gentle shaking at room temperature. If background counts on the filters were still high after this washing regime, further washes in 0.1 x SSC and 0.1 - 0.5% SDS at room temperature were performed. Where necessary, the stringency of the washing conditions was increased by raising the washing temperature to 50°C. Excess washing buffer was blotted from the filters which were then covered with Saran Wrap (to keep them damp). The wrapped filters were exposed to X-ray film (Kodak XAR-5) with intensifying screens (Dupont Lightning Plus) at -70°C. Densitometric scanning of the X-ray films was performed with an LKB Ultroscan XL scanning densitometer.

5.13 Repeated hybridisation of Northern blots

After autoradiography, filters were stripped of probe by washing in stripping buffer (5 mM Tris-HCl pH 8, 0.2 mM EDTA, 0.05% pyrophosphate, 0.1% Denhardts) at 65°C for 1 - 2 h. Filters were stored dry between Whatman filter paper until required for further hybridisations.

RESULTS

IL-3 enhances c-*fms* transcript expression in mouse bone marrow cells

Bone marrow cells were prepared from DBA/2 and A/J mice (method 2.1) and either lysed immediately for subsequent RNA extraction (method 5.4) or cultured at 1 x 10^6/ml in 100 U/ml IL-3. After 48 h culture, RNA was extracted from the non-adherent cells. WEHI-3B cells, which have previously been shown to express c-*fms* transcripts, were used as a positive control and L929 cells were used as a negative control. The yield of RNA from both fresh and cultured bone marrow cells was very low in comparison with that recovered from cell lines (Table 5.1).

20 µg of each RNA sample were run on a 1% agarose-formaldehyde gel (method 5.11). After overnight transfer to nitrocellulose, the filter was hybridised to a ^{32}P-labelled v-*fms* probe (method 5.12). The blot was later washed and rehybridised with a ubiquitin probe to control for RNA loading. WEHI-3B cells expressed c-*fms* transcripts of approx 3.8 kb but no specific message was detected in L929 cells (Fig 5.1). Both the fresh bone marrow samples contained c-*fms* RNA (approx. 3.8 kb) although the level of these transcripts was slightly higher in A/J than in DBA/2 bone marrow cells. In both strains, 48 h treatment with IL-3 greatly enhanced c-*fms* message levels (Fig 5.1).

Fig. 5.1 Effect of IL-3 on c-*fms* transcript levels in bone marrow cells

Nothern analysis of RNA (20 µg per lane)from A/J and DBA/2 bone marrow cells using a v-*fms* probe. RNA was pepared by ultracentrifugation through caesium chloride (method 5.4).

Lanes: (1) L929 cells, (2) WEHI-3B cells,(3) freshly isolated A/J bone marrow cells, (4) A/J bone marrow cells cultured with IL-3 for 48h, (5) freshly isolated DBA/2 bone marrow cells, (6) DBA/2 bone marrow cells cultured with IL-3 for 48h.

Rehybridisation with a ubiquitin probe showed equivalent amounts of RNA in each lane (data not shown).

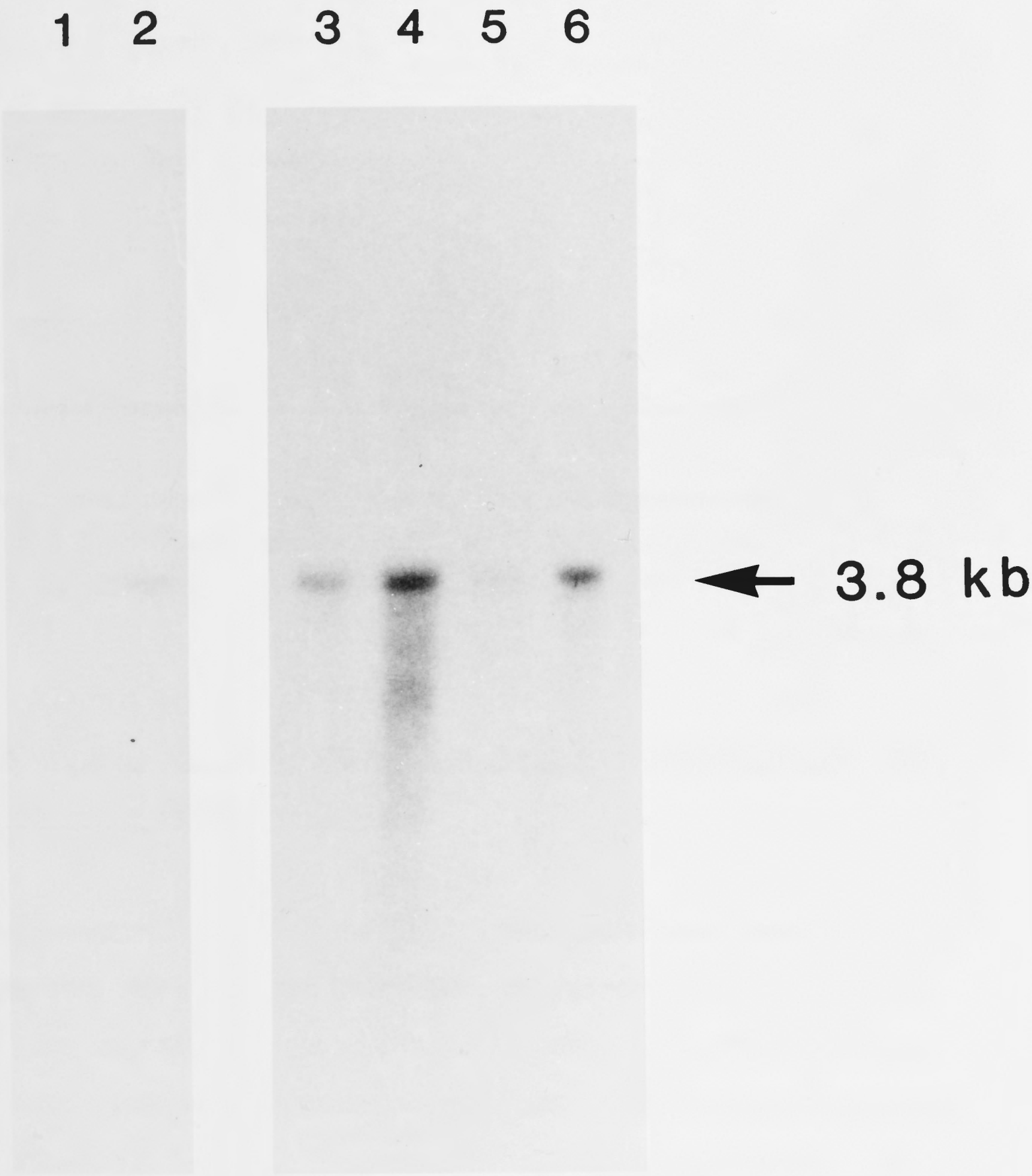

1 2
3 4 5 6
3.8 kb

Table 5.1 Yield of RNA from bone marrow cells and cell lines

SAMPLE	RNA YIELD ($\mu g/10^8$ cells)
DBA/2 Bm (freshly isolated)	40
DBA/2 Bm (cultured with IL-3)*	45
A/J Bm (freshly isolated)	25
A/J Bm (cultured with IL-3)*	28
L929	1100
WEHI-3B	780

RNA isolated by ultracentrifugation through caesium chloride (method 5.4). *Culture conditions as described in text.
Bm, bone marrow.

Time course of IL-3 induced c-*fms* expression in A/J and DBA/2 bone marrow cells

In an attempt to improve the RNA yields from bone marrow cells, a single step RNA isolation procedure was used to prepare RNA for this time course. The limited handling involved in this technique is claimed to minimize degradation and loss of RNA (Chomczynski and Saachi, 1987).

Freshly isolated bone marrow cells from A/J and DBA/2 mice were either lysed immediately for subsequent RNA extraction (method 5.5) or cultured at 1 x 10^6/ml in 100 U/ml IL-3. After 2, 6, 12, 24,

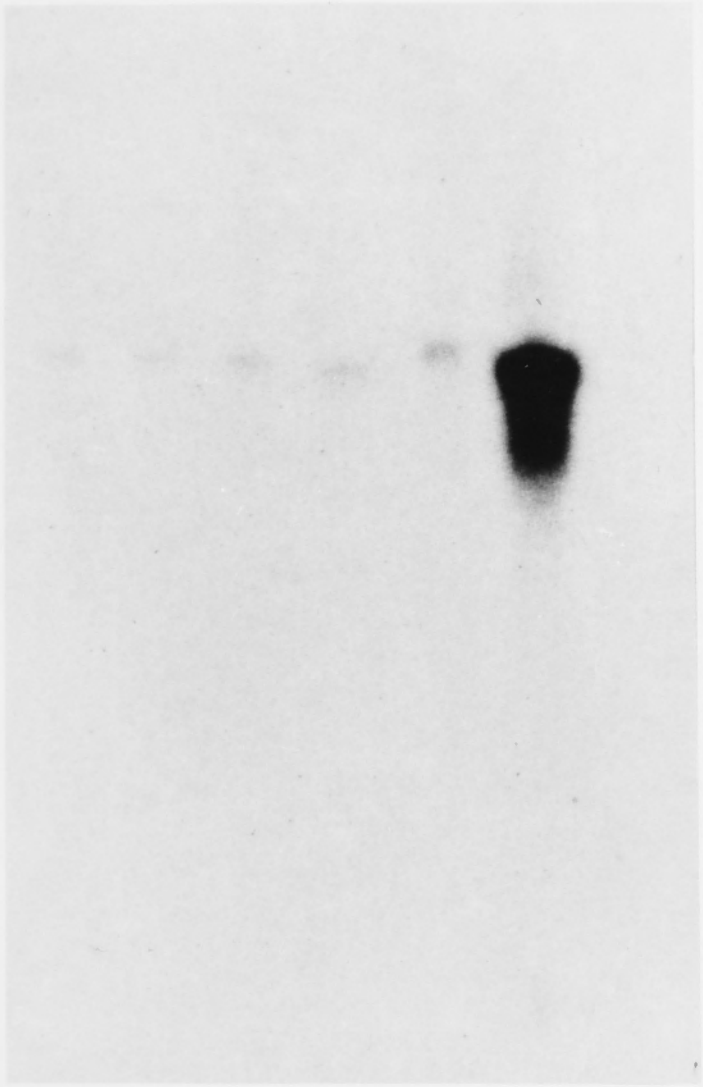
or 48 h culture, total RNA was extracted from the non-adherent cell population (method 5.5). Polyadenylated RNA was prepared (method 5.6) from the macrophage-like cell line PU5, to serve as a positive control. L929 RNA was used as a negative control. The yields of RNA from 1 x 10^8 bone marrow cells ranged from 80-150 µg using this single step method. 20 µg total RNA from each bone marrow sample was analysed by Northern blotting (method 5.11) and hybridisation with the v-*fms* probe (method 5.12) The blots were later washed and rehybridised with the ubiquitin probe to control for RNA loading.

Hybridisation with the ubiquitin probe revealed that the amounts of A/J bone marrow cell RNA loaded for each time point were not equal. However,the low levels of c-*fms* RNA expression detected in freshly isolated A/J bone marrow cells, was obviously enhanced after 24 h exposure to IL-3 (Fig 5.2). Exposure to IL-3 for a total of 48 h did not further enhance c-*fms* transcript levels (data not presented), suggesting that the maximal effect had occurred. A similar result was obtained with DBA/2 bone marrow cells although some enhancement of c-*fms* message levels was observed after only 12 h culture with IL-3 (Fig 5.3). A/J and DBA/2 cells cultured in the absence of IL-3 for 24 h did not show enhanced c-*fms* message levels (data not presented). PU5 mRNA contained high levels of c-*fms* transcripts of a similar size to those present in bone marrow. The sharp bands of c-*fms* message detected on these Northern blots indicates the integrity of the samples prepared using the single step method. Therefore, this method was used routinely for future RNA preparations.

Fig 5.2 Time course of IL-3-induced c-*fms* expression in A/J bone marrow cells

Northern analysis of RNA (20 µg per lane) from A/J bone marrow cells after culture with IL-3 for 0 h (lane 1), 2 h (lane 2), 6 h (lane 3), 12 h (lane 4), or 24 h (lane 5). 5 µg poly (A)+ RNA from PU5 cells (lane 6) and 20 µg RNA from L929 cells (lane 7) were used as controls. RNA was isolated from bone marrow cells by a single step procedure (method 5.5). Blot was hybridised first with a v-*fms* probe (A) and then with a ubiquitin probe to control for RNA loading (B).

A. 1 2 3 4 5 6 7



3.8 kb

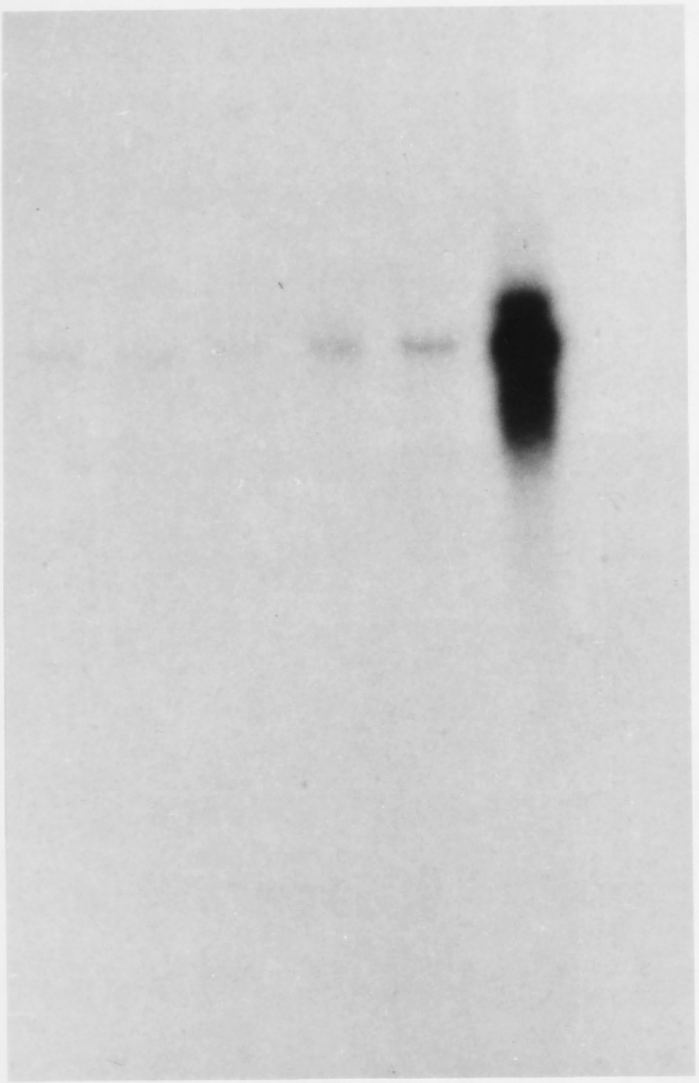
B. 1 2 3 4 5 6 7

1.4 kb

Fig 5.3 Time course of IL-3-induced c-*fms* expression in DBA/2 bone marrow cells

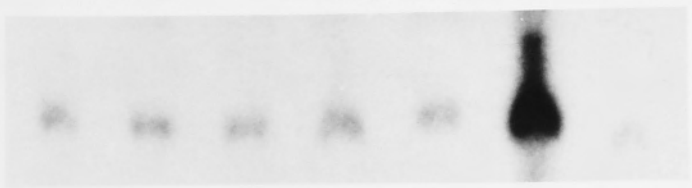
Northern analysis of RNA (20 µg per lane) from DBA/2 bone marrow cells after culture with IL-3 for 0 h (lane 1), 2 h (lane 2), 6 h (lane 3), 12 h (lane 4), or 24 h (lane 5). 5 µg poly (A)+ RNA from PU5 cells (lane 6) and 20 µg RNA from L929 cells (lane 7) were used as controls. RNA was isolated from bone marrow cells by a single step procedure (method 5.5). Blot was hybridised first with a v-*fms* probe (A) and then with a ubiquitin probe to control for RNA loading (B).

A. 1 2 3 4 5 6 7



3.8 kb

B. 1 2 3 4 5 6 7



1.4 kb

Effect of IL-3 on c-*fms* expression in bone marrow-derived macrophages

Macrophages produced from A/J and DBA/2 bone marrow cells (method 5.2) were either lysed immediately for subsequent RNA extraction (method 5.5) or were rinsed with fresh media and cultured for 12 or 24 h with 200 U/ml IL-3 before lysis. 20 μg of total RNA from each macrophage sample was analysed by Northern blotting (method 5.11) and hybridisation with the v-*fms* probe (method 5.12). PU5 mRNA and L929 total RNA were used as positive and negative controls respectively.

A high level of c-*fms* message was found in all macrophage samples, equivalent to approximately half the message expressed in 5 μg of PU5 mRNA (Fig 5.4a). Assuming that selection of polyadenylated RNA enriches message levels at least 20 times, the expression of c-*fms* RNA by the macrophage samples is approximately 2.5 times that expressed by the PU5 cell line. Incubation with IL-3 for 12 or 24 h did not result in a detectable enhancement in the level of c-*fms* message expressed by bone marrow-derived macrophages (Fig 5.4a).

Effect of IL-3 plus M-CSF on c-*fms* expression

Freshly isolated A/J bone marrow cells were either lysed immediately for subsequent RNA extraction (method 5.5) or cultured at 1 x 10^6/ml in IL-3 (200U/ml), M-CSF (200U/ml) or IL-3 plus M-CSF for 72 h (method 5.1). After culture, RNA was extracted from both non-adherent and adherent cells (method 5.5). Bone

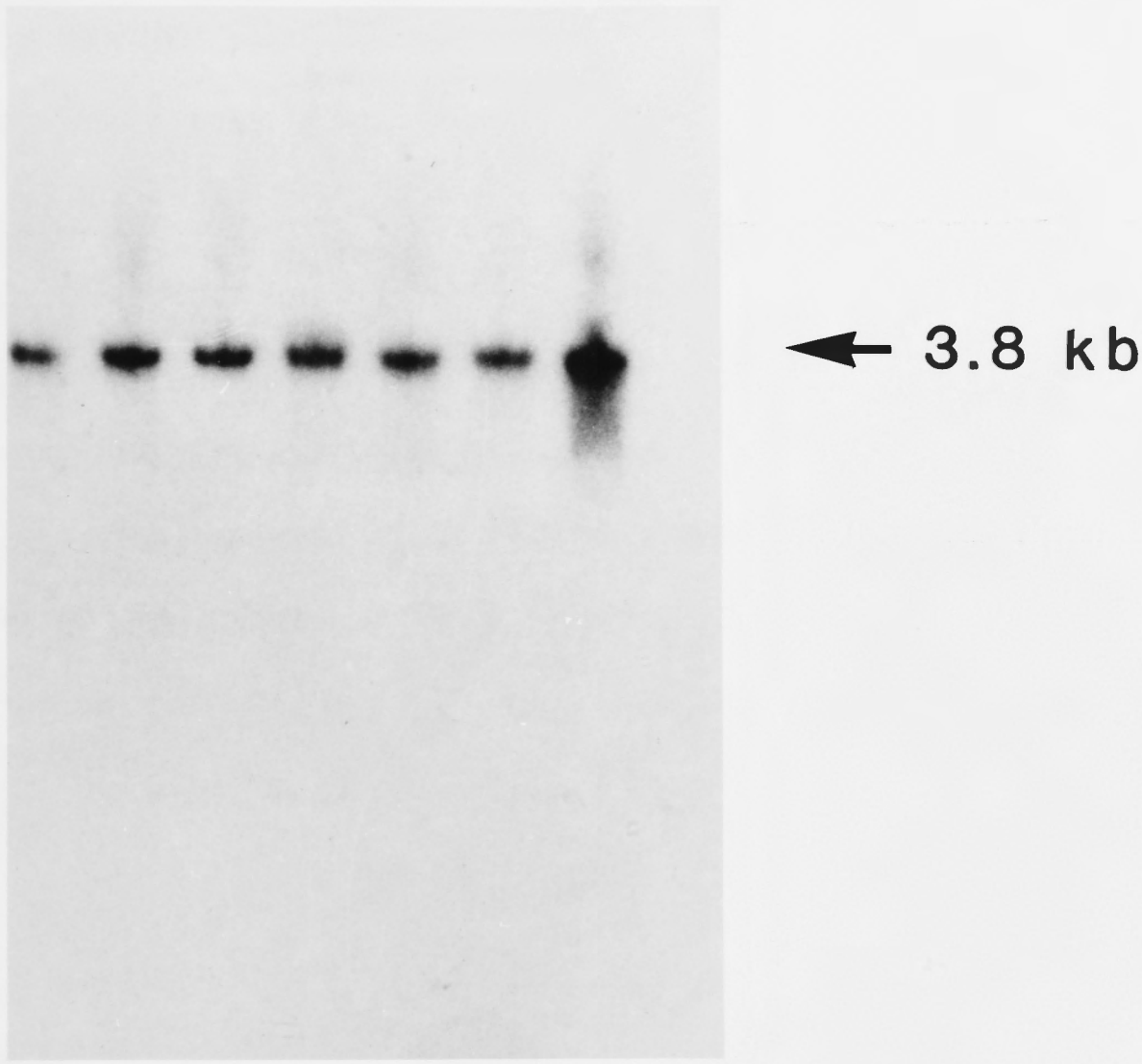
Fig 5.4a <u>C-*fms* transcript levels in bone marrow-derived macrophages before and after culture with IL-3</u>

Northern analysis of RNA (20 µg per lane) from A/J (lanes 1 - 3) and DBA/2 (lanes 4 - 6) bone marrow-derived macrophages using a v-*fms* cDNA probe. RNA was extracted from bone marrow-derived macrophages after culture in IL-3 for 0 h (lanes 1and 4), 12 h (lanes 2 and 5) or 24 h (lanes 3 and 6). 5 µg poly (A)+ RNA from PU5 cells (lane 7) and 20 µg RNA from L929 cells (lane 8) were used as controls. Rehybridisation with a ubiquitin probe showed equivalent amounts of RNA in each of lanes 1 - 6 (data not shown).

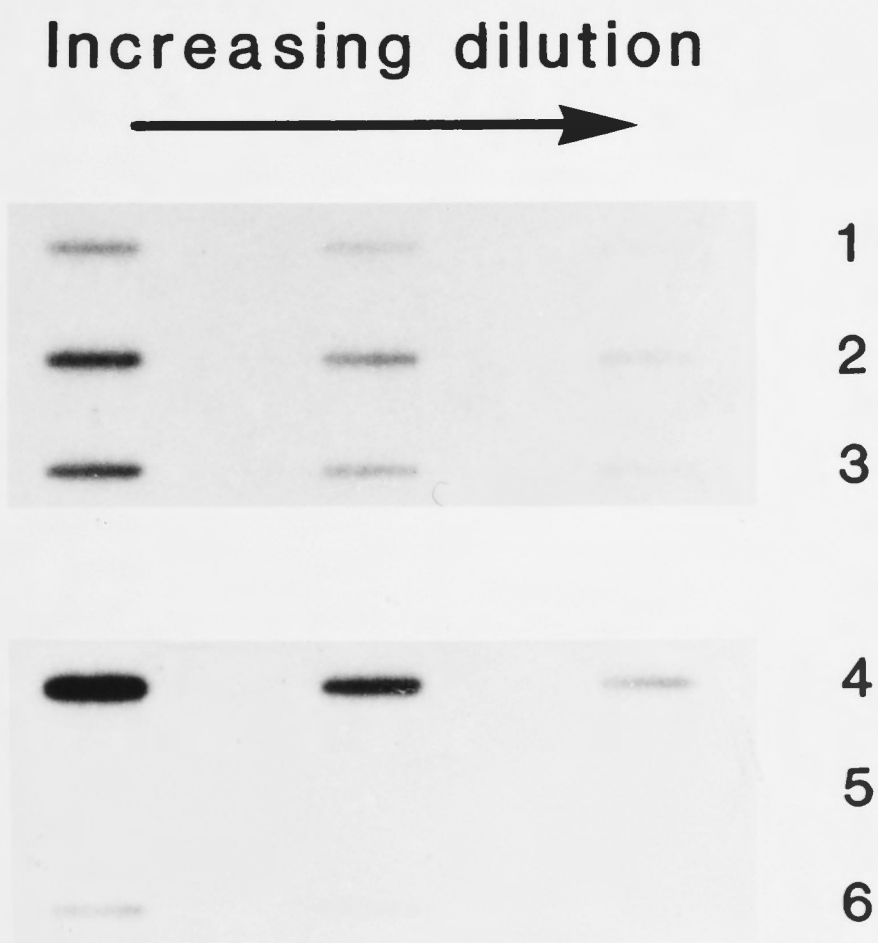
Fig.5.4b <u>Effect of IL-3, M-CSF, and IL-3 plus M-CSF on c-*fms* transcript levels in A/J bone marrow cells.</u>

Slot blot analysis of RNA from A/J bone marrow cells after 48 h culture with M-CSF (lane 2), IL-3 (lane 3) or IL-3 plus M-CSF (lane 4). RNA samples from freshly isolated A/J bone marrow cells (lane 1), L929 cells (lane 5), and WEHI-3B cells (lane 6) were used as control. RNA was blotted in serial triplicate dilutions giving equivalents of 10 µg, 3.3 µg and 1 µg and hybridised with a v-*fms* cDNA probe.

A.



B.



marrow RNA was applied to a slot blot (method 5.10) along with positive and negative control RNA from WEHI-3B and L929 cells respectively. The slot blot was then hybridised with the v-*fms* probe (method 5.12).

A/J bone marrow cells cultured in either IL-3 or M-CSF expressed somewhat higher levels of c-*fms* transcripts than did fresh A/J bone marrow. Furthermore, c-*fms* RNA expression was strikingly enhanced by culture in the combination of factors (Fig 5.4b). Densitometer analysis revealed that IL-3 plus M-CSF increased c-*fms* transcript levels synergistically (Table 5.2).

Table 5.2 Expression of c-*fms* transcripts in A/J bone marrow cells after treatment with IL-3, M-CSF or IL-3 + M-CSF

GROWTH FACTOR(S)	Relative Levels of C-*fms* Transcripts
M-CSF	1.8
IL-3	1.5
IL-3 + M-CSF	4.1
Control	1.0

Values were determined by densitometric scanning of the autoradiogram represented in Fig 5.4b. The values are expressed as the signal density relative to the control (RNA isolated from freshly isolated A/J bone marrow cells)

DISCUSSION

These results demonstrate the presence of c-*fms* transcripts at high levels in bone marrow-derived macrophages. C-*fms* transcripts are also easily detectable in freshly isolated bone marrow cells. This was not unexpected because transcripts of c-*fms* RNA have previously been detected in the myelomonocytic cell line, WEHI-3B (Gonda and Metcalf,1984), purified mouse granulocyte-macrophage progenitor cells (Bicknell et al.,1988), and in human bone marrow, peripheral blood mononuclear cells and haemopoietic cell lines induced along the monocyte differentiation pathway (Nienhuis et al.,1985; Sariban et al.,1985).

The present study also reveals that exposure to IL-3 enhances the expression of c-*fms* RNA transcripts in non-adherent bone marrow cells. Although this increase in mRNA transcript levels is not necessarily correlated with an elevation in levels of functional M-CSF receptors, this interpretation would be consistent with a previous study in which IL-3 was shown to increase the binding of [^{125}I]-M-CSF to non-adherent bone marrow cells (Bartelmez et al.,1985). In the latter study, IL-3 was shown to induce the *de novo* appearance of blast cells bearing low levels of M-CSF receptor and the authors suggested that this effect was most likely due to the proliferation of IL-3 responsive cells which already bear M-CSF receptors. However, the magnitude of enhancement of c-*fms* RNA levels was similar in A/J and DBA/2 cells even though IL-3-induced proliferation occurs only in the DBA/2 cultures. Together, these results suggest that IL-3 induces the appearance of M-CSF receptors on M-CSF receptor negative cells.

An elevated expression of M-CSF receptors has been correlated with increased proliferative activity in M-CSF (Chen and Clark,1986). In contrast, in functionally mature macrophages, M-CSF receptor expression is suppressed and there is a concommitant reduction in the proliferative activity of these cells. Thus, the increase in receptor expression in non-adherent cells could account for the enhanced M-CSF responsiveness observed in the A/J system in the presence of IL-3. Elevated expression of M-CSF receptors has also been correlated with commitment to the macrophage lineage (Bartelmez et al.,1985). However, the increased range of colony types produced by lower M-CSF concentrations (Chapter 4) suggests that lineage definition may also be influenced by the rate of occupancy or turnover of M-CSF receptors.

In the present study, culture with IL-3 did not enhance the levels of c-*fms* RNA expression in bone marrow-derived macrophages. However, in a report by Chen and Clark (1986), IL-3 was shown to increase the binding of [^{125}I]-M-CSF to peritoneal exudate macrophages. The most likely explanation for this apparent contradiction in results is the difference between the macrophage populations studied. The macrophages in the present study had been derived by culture for 7 days in a source of M-CSF. Such conditions have been shown to enhance the expression of M-CSF receptors not only by expansion of M-CSF receptor bearing cells but also by increasing receptor levels on individual cells (Bartelmez et al.,1985). Possibly then, c-*fms* expression was already maximally induced in the bone marrow-derived macrophages before exposure to IL-3. Indeed, Northern analysis revealed that the level of c-*fms*

transcripts in these macrophages was approximately 2.5 times the level present in the macrophage cell line PU5.

Both M-CSF and IL-3 enhanced the expression of c-*fms* RNA in 3 day A/J bone marrow cell cultures. The greater than additive effect of the combination of factors on c-*fms* transcript levels suggests that the induction of c-*fms* RNA by IL-3 results in the recruitment of more A/J bone marrow cells into an M-CSF responsive pool. This is in agreement with the previous observation that the presence of IL-3 increases the number of colonies produced by A/J bone marrow cells in soft agar culture with M-CSF (Chapter 4). The proposed antagonistic effect of IL-3 on M-CSF driven differentiation (see discussion Chapter 4), may also contribute to high c-*fms* expression in the presence of both factors by delaying the appearance of functionally mature macrophages.

The results presented in this and the previous chapter suggest that IL-3 synergises with M-CSF primarily by enhancing the expression of M-CSF receptors. An alternative hypothesis was put forward by Walker and associates (1985) in their report demonstrating that the binding of IL-3 to its receptor temporarily down-modulates M-CSF receptor expression by BALB/c bone marrow cells. The authors proposed that the ability of IL-3 to stimulate the proliferation and differentiation of cells of the macrophage lineage and to synergise with M-CSF might be explained by this down-modulation and thus activation of M-CSF receptors. However, since the down modulation of M-CSF receptors is proposed to evoke cellular proliferation, it is unlikely that such a response occurs after the binding of IL-3 to its receptors on A/J cells. Thus the relevance of IL-3-induced

receptor down-modulation for the synergistic activity of IL-3 is questionable.

The Northern analyses reported in this chapter revealed that the size of the c-*fms* RNA transcript in mouse bone marrow cells, bone marrow-derived macrophages, WEHI-3B and PU5 cells is approximately 3.8 kb and is therefore similar in size to the transcripts found in mouse placental tissue (Muller et al.,1983). An analysis of a variant of the WEHI-3B cell line, WEHI-3B (D^+), which has retained an ability to differentiate to macrophages or granulocytes in the presence of granulocyte (G)-CSF, revealed 2 c-*fms* transcripts of 8.4 kb and 4.1 kb (Gonda and Metcalf., 1984). The smaller of these transcripts probably corresponds to the transcript we observed in WEHI-3B (D^-) cells. Although the evidence is circumstantial, it is tempting to speculate that the absence of the larger transcript is correlated with the absence of differentiative capacity in the WEHI-3B (D^-) subline.

Chapter 6
Study of the interactions of IL-3 with IL-4, IL-1, and Erythropoietin

INTRODUCTION

As discussed in Chapter 1, IL-3 interacts with erythropoietin (Epo) to enhance erythroid burst formation *in vitro*. In 1978, Iscove observed that early erythroid progenitors BFU-E, could be maintained for several days in the absence of exogenously added Epo but maximal erythroid burst formation required both IL-3 and Epo during later stages of culture. In addition, the more mature erythroid progenitors produced small erythroid colonies in the presence of Epo alone. These observations led to the 2-stage model of humoral regulation of erythropoiesis in which early progenitors (BFU-E) possess receptors for IL-3 but not Epo whereas cells later in the maturation progression (CFU-E) have lost IL-3 receptors and acquired receptors for Epo.

The results of subsequent studies however, suggest that this model may be oversimplified. For example, Epo apparently acts directly on early progenitor cells since this factor is required for the generation of CFU-E in long term bone marrow cultures (Eliason et al.,1979) and the presence of Epo during preincubation of total bone marrow increases the ratio between BFU-E and GM progenitors (Van Zant and Goldwasser,1979). Furthermore, under certain circumstances, IL-3 can apparently drive the complete sequence of events leading to the mature red cell (Goodman et al.,1985) although the failure to detect IL-3 receptors on nucleated erythroid cells suggests that IL-3 does not act directly on these cells (Nicola and Metcalf,1986). Thus there is some controversy regarding the relative importance of IL-3 and Epo and the

synergistic interaction between these factors for the maintenance of erythropoiesis.

IL-3 displays a different kind of interaction with IL-1 and IL-4. Although the latter 2 factors have little or no capacity to stimulate haemopoietic colony formation, they both enhance the effects of other factors. In combination with M-CSF, IL-1 enhances the development of primitive haemopoietic cells apparently by inducing the expression of M-CSF receptors normally expressed only on more mature cells (Bartelmez and Stanley,1985). IL-1 also enhances the response of early haemopoietic progenitors to IL-3 (Stanley et al.,1986). IL-4 enhances the effects of IL-3, Epo, G-CSF, and M-CSF (Rennick et al.,1987). In combination with IL-3, IL-4 enhances the growth of mast cell lines and enhances mast cell colony formation (Smith and Rennick, 1986; Rennick et al.,1987).

To further investigate the synergistic interactions between IL-3 plus Epo, IL-3 plus IL-1 and IL-3 plus IL-4, the effect of these combinations of factors was assayed in cultures of IL-3-responsive (DBA/2) and IL-3-hyporesponsive (A/J) bone marrow cells.

MATERIALS AND METHODS

CHEMICALS AND GROWTH FACTORS

Chemicals and growth factors used were as described in Chapter 2 unless otherwise stated. IL-1 (lot 14993-74/102) was donated by Hofman La Roche, Nutley, New Jersey, USA. Epo was obtained from Snow Brand Milk Products Co. Ltd., Tochigi, Japan. IL-4 was obtained from Immunex Corp., Seattle, USA.

METHODS

6.1 Soft agar colony assays

Colony assays were performed as described in method 2.3. The factors added were IL-3 (200 U/ml), IL-4 (50 U/ml), and IL-1 (25 U/ml). Control assays contained GM-CSF (50 U/ml). Assays were incubated for 7 days before scoring colony numbers.

6.2 Methylcellulose colony assays

Methylcellulose assays were set up essentially as described for soft agar colony assays (method 6.1) except that the culture medium consisted of 0.8% methylcellulose (Fluka) / 10^{-4} M 2-mercaptoethanol / 1% BSA / 30% FCS in Iscove's Modified Dulbecco's Essential Medium (Flow Laboratories). The factors added were IL-3 (200 U/ml), IL-1 (25 U/ml), Epo (2 U/ml) or combinations of these factors. Control assays contained GM-CSF (50 U/ml). After 12 days incubation, gels were placed on

microscope slides, then fixed in 5% glutaraldehyde in PBS (6 minutes), rinsed in distilled water (6 minutes)and dried at 37°C (approximately 30 minutes). Fixed gels were stained with 1% benzidine in methanol (2 minutes) then drained and placed in 2.5% H_2O_2 in 70% ethanol (1 minute). Slides were then washed in water dried, counterstained with Mayer's haematoxylin (5 minutes) and finally washed again with water.

RESULTS

Synergism between IL-3 and IL-4

To investigate the synergistic interaction between IL-3 and IL-4, the effect of this combination of growth factors was assayed in soft agar cultures of A/J and DBA/2 bone marrow cells (method 6.1). IL-4 alone failed to stimulate colony formation by A/J or DBA/2 bone marrow cells. However, when assayed in combination with IL-3, the presence of IL-4 significantly ($p<0.001$ according to the Students t test) enhanced the total number of colonies produced by DBA/2 cells (Table 6.1). A synergistic interaction between IL-3 and IL-4 was not seen in A/J bone marrow cell cultures.

Table 6.1 Strain-dependent synergism between IL-4 and IL-3.

	COLONY NUMBER			
STRAIN	IL-3	IL-4	IL-3 + IL-4	GM-CSF
DBA/2	26 ± 6	0	37 ± 4	11 ± 2
A/J	0	0	0	13 ± 2

DBA/2 and A/J cells were plated in colony assays with IL-3 (200 U/ml), IL-4 (50U/ml) or IL-3 plus IL-4. Cultures containing GM-CSF were set up in parallel. Data represents the mean ± SD of the number of colonies in triplicate cultures.

Synergistic interactions between IL-3, Epo and IL-1.

The effect of IL-3, Epo and IL-1 on erythroid cell production was examined using methylcellulose cultures (method 6.2). These growth factors were assayed alone and in all combinations on A/J and DBA/2 bone marrow cells. In DBA/2 bone marrow cell cultures, IL-1 did not significantly enhance the number of colonies produced in response to IL-3 or IL-3 plus Epo (Table 6.2). Furthermore, IL-1 and Epo, alone or in combination, failed to support colony formation in these cultures. Erythroid cells were produced only in the presence of both IL-3 and Epo (Table 6.2). IL-3, IL-1 and Epo, alone or in combination, failed to support colony formation by A/J bone marrow cells in methylcellulose cultures. Similarly in soft agar cultures (method 6.1), IL-3, IL-1, and IL-3 plus IL-1 failed to stimulate colony production by A/J bone marrow cells (data not shown).

Table 6.2 Effect of IL-3, Epo, and IL-1 on erythroid colony formation

	COLONY NUMBER			
FACTOR	DBA/2		A/J	
IL-3	15 ± 2	(-)	0	(-)
IL-1	0	(-)	0	(-)
Epo	0	(-)	0	(-)
IL-3 + IL-1	18 ± 2	(-)	0	(-)
IL-3 + Epo	17 ± 3	(18%)	0	(-)
IL-1 + Epo	0	(-)	0	(-)
IL-3 + IL-1 + Epo	21 ± 3	(24%)	0	(-)
GM-CSF	11 ± 3	(-)	12 ± 5	(-)

A/J and DBA/2 bone marrow cells were plated in methylcellulose colony assays with IL-3 (200 U/ml), IL-1 (25 U/ml) or Epo (2 U/ml) or combinations of these factors. Control assays contained GM-CSF (50 U/ml). Data represents the mean ± SD of the number of colonies in triplicate cultures. In parentheses are the number of colonies containing erythroid cells (as determined by benzidine staining) expressed as a percentage of the total colony count.

DISCUSSION

In soft agar cultures, IL-4 enhanced IL-3-dependent colony formation by DBA/2 bone marrow cells. This is consistent with previous studies revealing that IL-3 and IL-4 act synergistically to support mast cell proliferation (Smith and Rennick,1986). In a recent report, Rennick and colleagues reported that IL-4 suppresses IL-3-dependent colony formation by a purified population of multipotential progenitor cells (Thy $^{lo+}$). The results presented here do not necessarily contradict this observation because Thy $^{lo+}$ cells represent a relatively small proportion of the colony forming cells present in total bone marrow.

IL-3 did not act synergistically with IL-4 or Epo to produce colonies in A/J bone marrow cell cultures. Thus, these synergistic interactions are apparently inseparable from the mitogenic activity of IL-3. Assuming that IL-3 and Epo do not synergise *in vivo* in A/J mice, an alternative pathway must exist to allow production of erythroid cells in this strain. In this regard, Iscove and associates (1988) recently identified a soluble synergistic activity which cooperates with IL-3 and Epo in stimulating the growth of mixed erythroid colonies from primitive precursors. In addition, the direct surface-surface interactions observed between macrophages and erythroblasts in long term marrow cultures (Lichtman,1981) may be important for the maintenance of erythropoiesis in vivo.

No synergistic interaction between IL-1 and IL-3 was detected in semi-solid cultures of either A/J or DBA/2 bone marrow cells. In

contrast, previous work has shown that the combination of IL-1 and IL-3 stimulates a population of high proliferative potential colony forming cells (Stanley et al.,1986, McNiece et al.,1987). However, the latter studies used bone marrow cells from mice treated with 5-FU to enrich for non-cycling early progenitor cells. Possibly then, the cell population responsive to IL-1 plus IL-3 is too small to be detected in colony assays using normal bone marrow cells.

In conclusion, the data presented in this Chapter suggest that IL-3 does not interact with IL-4 or Epo in the A/J system. Therefore these interactions are probably not crucial for the development and maintenance of the haemopoietic system Further experiments, using purified early haemopoietic progenitors, are required to assess whether IL-1 interacts normally with IL-3 in the A/J system.

Chapter 7

Effect of IL-3 on the expression of growth factor message by haemopoietic cells

INTRODUCTION

In addition to its direct proliferative and synergistic effects, IL-3 may influence haemopoiesis indirectly by inducing the synthesis of other haemopoietic factors. In support of this proposal, there are numerous examples of the induction of one haemopoietic factor by another. In both man and mouse, M-CSF stimulates the release of G-CSF and/or GM-CSF from macrophages (Metcalf and Nicola,1985; Warren and Ralph,1986). IL-1 stimulates G-CSF and GM-CSF from several different cell types *in vitro* including cultured human fibroblasts (Zucali et al.,1986) and endothelial cells (Broudy et al.,1987). Furthermore, GM-CSF stimulates expression of message for M-CSF by human monocytes (Horiguchi et al.,1987).

The experiments discussed in Chapter 3 revealed that media conditioned by A/J and DBA/2 bone marrow cells cultured in the presence of IL-3, contained a non-IL-3 colony stimulating activity. Therefore, IL-3 apparently induces endogenous growth factor production by mouse haemopoietic cells. The aim of the experiments discussed in the present chapter was to investigate this phenomenon further by analysing the effect of IL-3 on the expression of growth factor message by mouse haemopoietic cell lines, bone marrow-derived macrophages, and freshly isolated bone marrow cells. The effects of IL-3 on growth factor message levels in A/J and DBA/2 bone marrow cells were compared to assess whether this activity is linked to the proliferative activity of IL-3.

MATERIALS AND METHODS

CHEMICALS, GROWTH FACTORS AND PLASMIDS

Chemicals, growth factors and plasmids were as described in Chapter 5 unless otherwise stated. Plasmids containing full length or partial cDNAs for M-CSF, GM-CSF, G-CSF, and IL-4 were kindly donated by T. Rajavashisth (UCLA School of Medicine,USA), N. Gough and A. Dunn (Ludwig Cancer Institute for Cancer Research, Victoria), S. Nagata (Institute of Medical Science, University of Tokyo, Japan), and F. Lee (DNAX Institute of Molecular and Cellular Biology, CA, USA) respectively.

METHODS

7.1 Culture of 32D cl-23 cells

32D cl-23 cells were maintained in RPMI-5% FCS supplemented with 30% WEHI-3B CM (method 3.4). Immediately before use, the cells were washed three times by pelleting at 200 x g for 10 mins and resuspending in 50 ml fresh RPMI-5% FCS with either 50 U/ml IL-3 or 50 U/ml IL-2 (Jansenn, Belgium). After 3 days culture at 37°C in a humidified CO_2 atmosphere, cells were harvested and lysed as described in method 5.4

7.2 Preparation of control RNA

PU5 mRNA, WEHI-3B total RNA, and L929 total RNA were prepared as described in Chapter 5. In addition, total RNA was prepared (method 5.4) from a human bladder carcinoma cell line, 5637, which constitutively produces G-CSF (Welte et al.,1985) and from COS-1 monkey cells 3 days after transfection with the mouse IL-4 gene [COS (IL-4)]. 5637 cells were maintained in RPMI-10%FCS. COS-1 cells were maintained in DMEM-10%FCS. PU5 mRNA, and total RNA from L929 and COS(IL-4) cells were applied together as a mixed marker. 20 μg of this mixed marker contained 12.5 μg PU5 mRNA, 6 μg L929 RNA, and 1.5 μg COS(IL-4) RNA.

7.3 Probe preparation

The following restriction enzyme fragments were isolated (method 5.8) for use as probe templates; M-CSF, a 170 bp *Pst*1-*Eco* R1 fragment of the mouse M-CSF cDNA (Rajavashisth et al.,1987) inserted into the multiple cloning site of the pGEM-2 vector; GM-CSF, a 700bp *Hind*111-*Eco*R1 fragment of the mouse GM-CSF cDNA derived from plasmid PGM38 (Gough et al.,1984); G-CSF, a 600 bp *Eco*R1-*Sau*3A fragment of plasmid pHCS-1 containing 308 bp of human G-CSF cDNA (Nagata et al.,1986); IL-4, a 373 bp *Rsa*O fragment of the mouse IL-4 cDNA inserted into a modified pcDV1 vector (Lee et al.1986); IL-3, a 588 bp *Hinc*11-*Nco*1 fragment of the mouse IL-3 cDNA (Fung et al.,1985) derived from pILM 21 (Campbell et al.,1988). ^{32}P-labelled probes were prepared from isolated fragments as described in method 5.12. Ubiquitin probes were prepared as described in method 5.7.

7.4 Repeated hybridisation of Northern blots

Northerns were prepared, prehybridised, hybridised and washed as described in methods 5.11 and 5.12. Hybridisation with the G-CSF probe was carried out at 37°C to allow cross hybridisation between the human probe and mouse RNA (Tsuchiya et al.,1986) After autoradiography, filters were stripped of probe as described in method 5.13.

RESULTS

Expression of growth factor message by 32D cl-23 cells

20 µg total RNA (prepared using method 5.4)from 32D cl-23 cells cultured in IL-3 or IL-2 (method 7.1) was analysed by Nothern blotting (method 7.4). 5 µg PU5 mRNA, 1 µg COS(IL-4) RNA and 1 µg WEHI-3B RNA were used as positive controls for GM-CSF, IL-4, and IL-3 respectively. The blot was hybridised sequentially with labelled cDNA probes (prepared using method 7.3) for GM-CSF, IL-3, and IL-4.

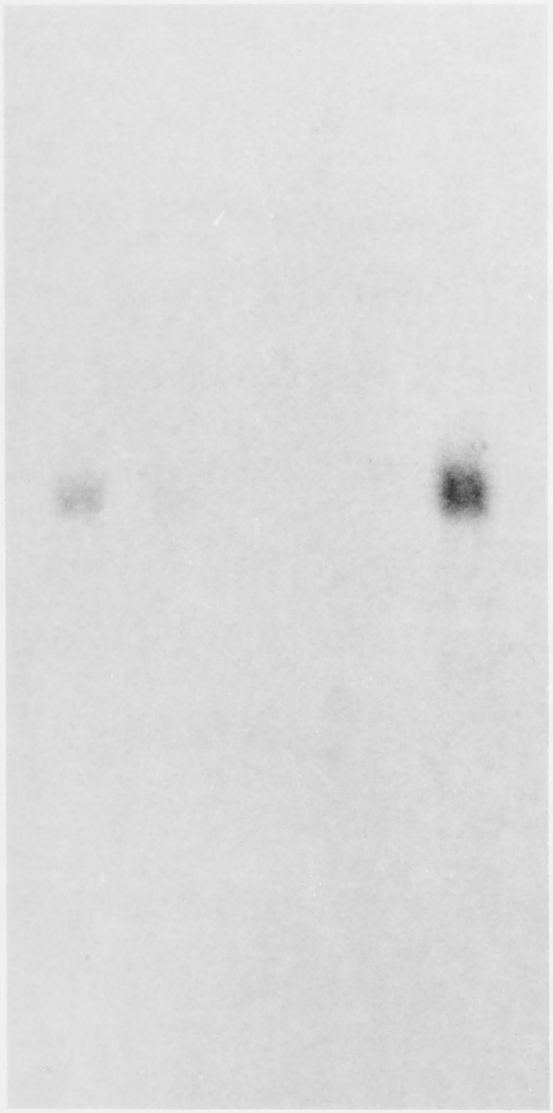
IL-4 transcripts of approximately 0.6 kb were just detectable in RNA from 32D cells maintained in IL-3 but not in RNA from 32D cells which had been transferred to IL-2 (data not shown). No GM-CSF or IL-3 transcripts were detected in these cells irrespective of the culture conditions.

The experiment was repeated using 20 µg poly (A)$^+$ RNA (prepared using method 5.6) from 32D cells cultured in IL-3 or IL-2. IL-4 transcripts were easily detectable in RNA from 32D cells maintained in IL-3 and were just detectable in RNA from 32D cells which had been transferred to IL-2 (Fig 7.1). Again no GM-CSF or IL-3 transripts were detected in 32D cells irrespective of the culture conditions.

Fig. 7.1 Expression of growth factor transcripts by 32D cl-23 cells cultured in IL-3 or IL-2

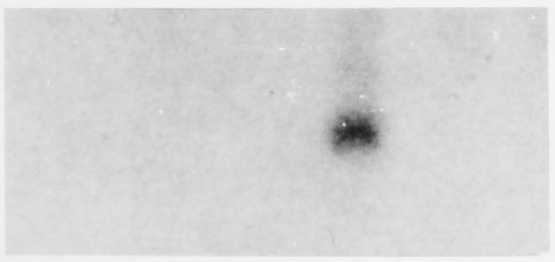
Northern analysis was performed with 20 μg of poly (A)+ RNA isolated from 32D cl-23 cells cultured in either IL-3 (lane 1) or IL-2 (lane 2). 1 mg WEHI-3B total RNA (lane 3), 5 μg PU5 poly (A)+ RNA (lane 4) and 1 mg COS(IL-4) total RNA (lane 5) were used for controls. The blot was hybridised with cDNA probes for (A) IL-4 (0.6 kb message), (B) GM-CSF (1.2 kb message), and (C) IL-3 (1.2 kb message). Rehybrisation with a ubiquitin probe showed equivalent amounts of RNA in lanes 1 and 2 (data not shown)

A. 1 2 3 4 5



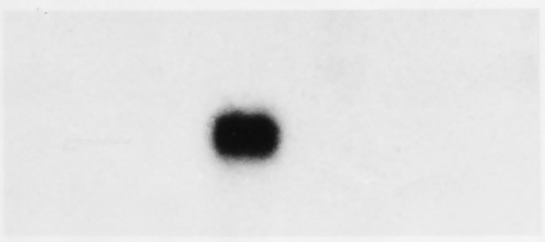
0.6 kb

B. 1 2 3 4 5



1.2 kb

C. 1 2 3 4 5



1.2 kb

Expression of growth factor message by bone marrow-derived macrophages

The preparation of RNA from bone marrow-derived macrophages was described in Chapter 5. RNA (20 μg) from A/J and DBA/2 bone marrow-derived macrophages after 0, 12, or 24 h culture in IL-3 was analysed by Northern blotting. 5637 RNA (20 μg) was used as a positive control for G-CSF. Mixed control (10 μg) containing RNA from PU5, L929, and COS(IL-4) cells (method 7.2) was used as a positive control for IL-4, GM-CSF and M-CSF. The blots were hybridised sequentially (method 7.4) with labelled cDNA probes (Prepared using method 7.3) for GM-CSF, G-CSF, and IL-4.

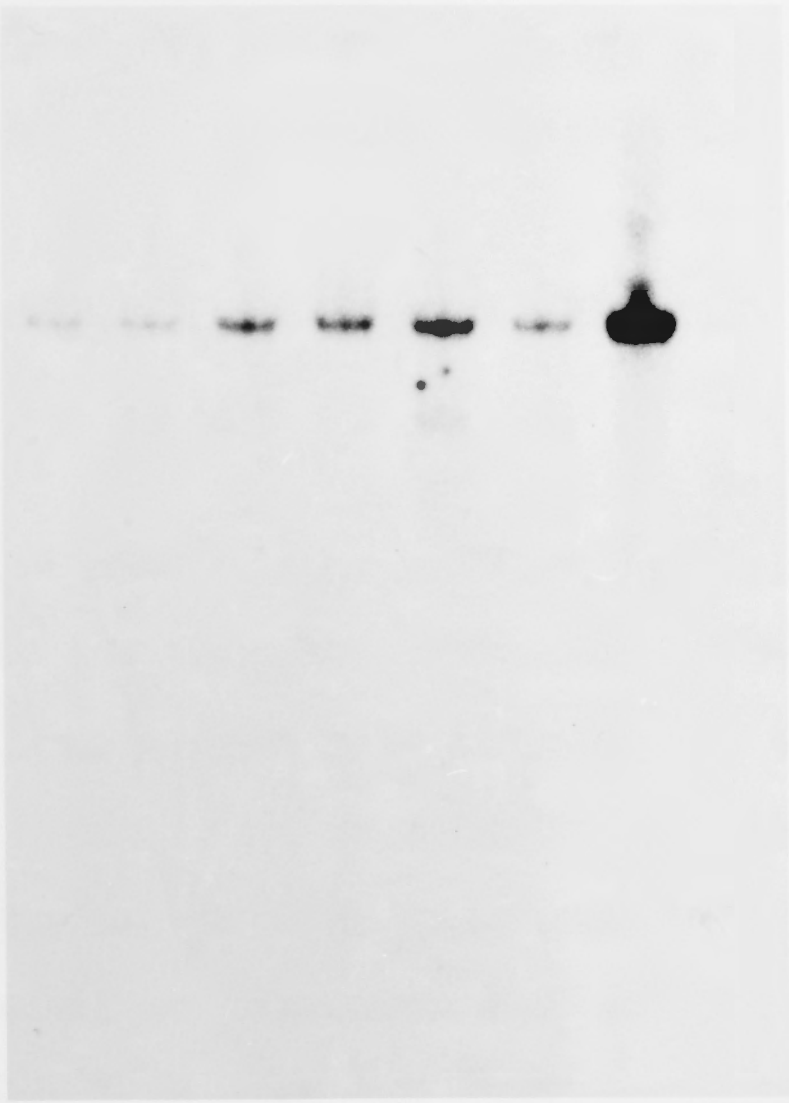
A/J bone marrow-derived macrophages contained M-CSF transcripts prior to culture in IL-3 and the level of these transcripts was augmented by 24 h culture in IL-3. DBA/2 bone marrow-derived macrophages prior to culture in IL-3 expressed M-CSF transcripts at higher levels than were detected in the corresponding A/J sample (Fig 7.2). In DBA/2 macrophages, M-CSF message levels were enhanced after only 12 h culture in IL-3 and had returned to control levels by 24 h. A repeat of this experiment confirmed the difference in the kinetics and magnitude of M-CSF transcript expression between A/J and DBA bone marrow-derived macrophages (data not shown). In both strains, the most abundant form of M-CSF mRNA is approximately 3.8 kb in size but another transcript of approximately 2.5 kb was also found.

Transcripts for G-CSF, GM-CSF, or IL-4 were not detected in bone marrow-derived macrophages either before or after culture with

Fig 7.2 Induction of M-CSF message in bone marrow-derived macrophages by culture with IL-3

Northern analysis of RNA (20 µg per lane) extracted from A/J (lanes 1 - 3) and DBA/2 (lanes 4 - 6) bone marrow-derived macrophages after culture in IL-3 for 0 h (lanes1 and 4), 12 h (lanes 2 and 5), or 24 h (lanes 3 and 6). Mixed marker RNA [6µg L929 RNA , 12.5 µg PU5 poly (A+) RNA and 1.5 µg COS (IL-4) RNA] and 5637 RNA (lane 8) were used as controls. The blot was hybridised with cDNA probes for (A) M-CSF (3.8 kb and 2.5 kb message), (B) GM-CSF (1.2 kb message), (C) G-CSF (1.6 kb message)and (D) IL-4 (0.6 kb message). Rehybridisation with a ubiquitin probe showed equivalent amounts of RNA in lanes 1 - 6 (data not shown).

A. 1 2 3 4 5 6 7 8



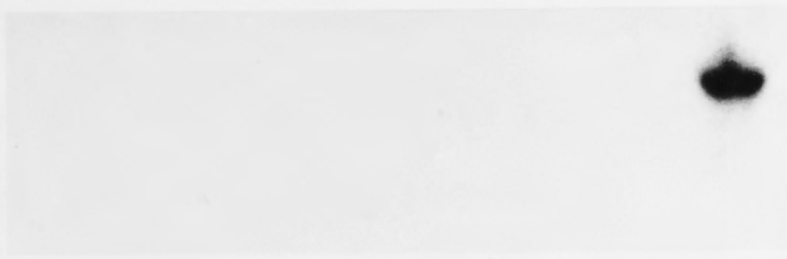
← 3.8 kb

← 2.5 kb

B. 1 2 3 4 5 6 7 8

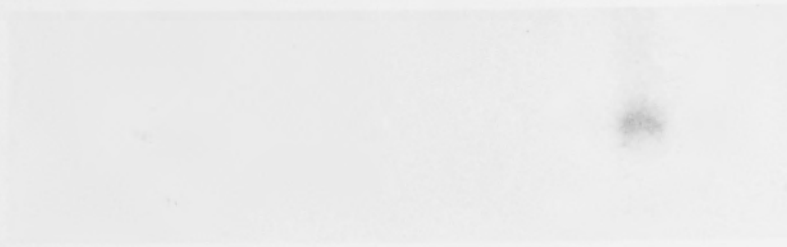
← 1.2 kb

C. 1 2 3 4 5 6 7 8



← 1.6 kb

D. 1 2 3 4 5 6 7 8



← 0.6 kb

IL-3 (Fig 7.2). Similarly, previous experiments failed to detect IL-3 message in such macrophages (data not presented).

Expression of growth factor transcripts in bone marrow cells

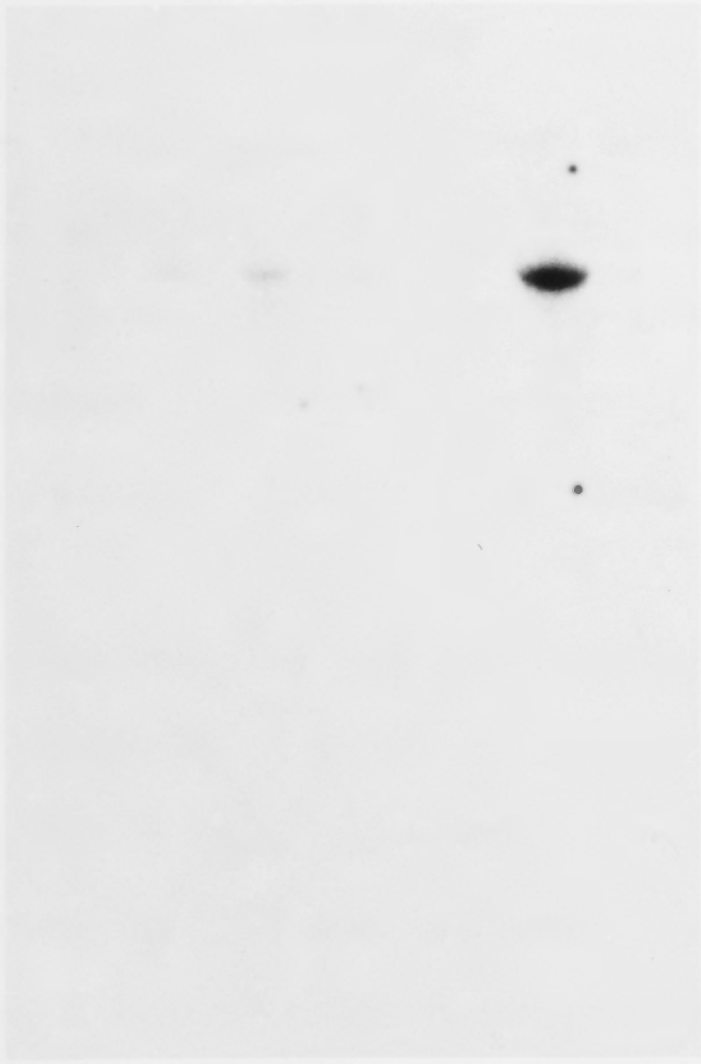
The preparation of bone marrow cell RNA samples used for these Northern analyses was described in Chapter 5. RNA (20 μg) from DBA/2 and A/J bone marrow cells cultured in IL-3 for 0, 2, 6, 12, or 24 h was analysed by Northern blotting (method 5.11). Control RNA samples were the same as those used for the analysis of growth factor transcript production by bone marrow-derived macrophages. The blots were hybridised sequentially (method 7.4) with cDNA probes for G-CSF, M-CSF, GM-CSF and IL-4 (probes prepared using method 7.3).

Low levels of M-CSF transcripts of 3.8 kb were detectable in all bone marrow samples. Following 6 h exposure to IL-3, the level of M-CSF message was transiently augmented, returning to control levels after 12 h of culture (fig 7.3) Transcripts for G-CSF, GM-CSF, and IL-4 were not detected in any of the bone marrow cell samples (data not shown). Similarly, in previous experiments, IL-3 transcripts have not been detected in bone marrow cells before or after exposure to IL-3 (data not shown).

Fig 7.3 Induction of M-CSF message in bone marrow cells cultured with IL-3

Northern analysis of RNA (20 µg per lane) extracted from (A) A/J and (B) DBA/2 bone marrow cells cultured in IL-3 for 0 h (lane 1), 2 h (lane 2), 6 h (lane 3), 12 h (lane 4) or 24 h (lane 5). Mixed marker RNA [6 µg L929 RNA, 12.5 µg PU5 poly (A)+ RNA and 1.5 µg COS (IL-4) RNA] (lane 7) and 5637 RNA (lane 8) were used as controls. The blots were hybridised with a cDNA probe for M-CSF (3.8 kb message). Rehybridisation with a ubiquitin probe showed equivalent amounts of RNA in each of lanes 1 - 5 (data not shown).

A. 1 2 3 4 5 6 7



← 3.8 kb

B. 1 2 3 4 5 6 7

← 3.8 kb

DISCUSSION

Although a number of haemopoietic growth factor cascades have been identified, the effect of IL-3 on growth factor production by haemopoietic cells has not been extensively examined. The results presented here however, clearly demonstrate that IL-3 modulates growth factor expression in haemopoietic cells.

32D cl-23 cells share many of the characteristics of mast cells; they proliferate in response to IL-3, are alcian blue positive and form basophil colonies in agar. However, 32D cl-23 cells also proliferate in response to IL-2 and are lyt1$^+$, suggesting that these cells may represent a branch point in haemopoiesis between the mast cell and lymphoid lineage. The observation that 32D cl-23 cells grown in IL-3 express IL-4 transcripts is consistent with a recent report demonstrating that various mast cell lines contain IL-4 message (Brown et al.,1987). The present study further demonstrates that when 32D cl-23 cells are transferred to medium supplemented with IL-2, the expression of IL-4 transcripts is considerably reduced. Therefore, IL-3 apparently induces IL-4 expression in these cells.

M-CSF transcripts were readily detectable in all bone marrow-derived macrophage RNA samples. The predominant transcript size was 3.8 kb but a low level of 2.5 kb transcripts were also present. These transcripts probably correspond to the 3.8 kb and 2.3 kb transcripts reported by Rajavashisth et al.(1987). In addition, Rajavashisth's group found 4.5 kb and 1.4 kb M-CSF transcripts.

The presence of M-CSF RNA in bone marrow-derived macrophages prior to exposure to IL-3 may indicate that M-CSF is constitutively produced by cells of the monocyte/macrophage lineage. Two studies of the human system support this notion: Horiguchi et al.(1986) detected low levels of M-CSF in human monocytes and Vellenga et al.(1988) observed that adherence of monocytes elevated the level of M-CSF transcripts expressed by these cells. Both these groups however, found that M-CSF transcript levels expressed in monocytes were barely detectable. In contrast, in the present study bone marrow-derived macrophages contained relatively high levels of M-CSF message, suggesting that the culture conditions used to produce these macrophages may have enhanced the expression of M-CSF RNA. Indeed, numerous studies indicate that M-CSF stimulates macrophages to release increased amounts of secretory factors. Furthermore, one of the secretory factors induced, interferon-γ (Moore et al.,1981), will itself induce synthesis of M-CSF by macrophages (Cosman,1987; Rambaldi et al.,1987). Therefore, M-CSF message expression in bone marrow-derived macrophages may be enhanced by an autostimulatory pathway. In support of this proposal, A/J bone marrow-derived macrophages, which respond poorly to interferon-γ (Hamilton et al.,1986), express considerably lower levels of M-CSF transcripts than do their counterparts derived from DBA/2 mice.

Exposure to IL-3 elevates the expression of M-CSF message by bone marrow-derived macrophages. This is in agreement with a report by Vellenga et al.(1988) who demonstrated an enhancement in M-CSF transcript levels in human monocytes exposed to IL-3. Although IL-3 elevated M-CSF RNA levels in both A/J and DBA/2

macrophages, the A/J cells required a longer exposure to IL-3 than did the DBA/2 cells. This may reflect either a strain-dependent difference in responsiveness to IL-3 or a difference in the interferon-induced activation state of the cells as discussed above.

In the present report, no evidence was found that IL-3 induced G-CSF, GM-CSF, or IL-4 production by bone marrow-derived macrophages. Similarly, Vellenga et al.(1988) found no enhancement of G-CSF or GM-CSF transcript levels in human monocytes exposed to IL-3. In contrast, Metcalf and Nicola (1985) reported that peritoneal exudate macrophages stimulated by IL-3, produced G-CSF. The apparent disparity between these results may be caused by the use of macrophages from different sources or by the relatively low sensitivity of Northern analysis compared with biological assays. Alternatively, the response of peritoneal macrophages, apparently elicited by IL-3, may have been caused at least in part by the high levels of endotoxin found in media used for that study (Metcalf and Nicola, 1985).

M-CSF transcripts are present in a number of mouse tissues including adult lung and heart (Rajavashisth et al.,1987) and fetal membranes and placenta (Azoulay et al.,1987). The detection of M-CSF transcripts in freshly isolated adult bone marrow further extends this list of tissues and is consistent with a report by Shadduck et al.(1983) demonstrating that M-CSF is produced in long term bone marrow cultures. Stromal cells are prime candidates as the cellular source of M-CSF and M-CSF RNA detected in bone marrow cultures. A fibroblast cell line, L929, expresses M-CSF

RNA and produces M-CSF constitutively (Stanley and Heard,1977.,Rajavashisth et al.,1987). M-CSF is present in the CM of 'adipocyte' cell lines (Lanotte et al.,1982) and, as discussed above, low levels of M-CSF RNA are detected in human monocytes. However, further work, perhaps using *in situ* hybridisation, is required to determine which types of bone marrow cell produce M-CSF *in vivo*.

IL-3 elevates the level of M-CSF message expressed in A/J and DBA/2 bone marrow cultures. This increase in message levels may be due either to the elevation of M-CSF expression by the constitutive producer cells or to induction of M-CSF RNA expression in another population of cells. Since IL-3 induces M-CSF RNA expression in cells of the monocyte/macrophage lineage, it is likely that these cells are the source of at least some of the induced transcripts observed in whole bone marrow. In this regard, the apparently transient nature of the response by non-adherant bone marrow cells may be due to adherence of the responsive macrophage population.

Irrespective of the nature of the producer cell, the presence of RNA transcripts for M-CSF but not G-CSF, GM-CSF or IL-4 in A/J and DBA/2 bone marrow cells cultured in IL-3 indicates that M-CSF probably accounts for the non-IL-3 endogenous colony stimulating activity found in the media conditioned by these cells. There is no apparent difference between A/J and DBA/2 cells in the magnitude of the induction of M-CSF RNA by IL-3. Therefore, the M-CSF-inducing activity is apparently separable from the proliferative activity of IL-3.

Chapter 8

General Discussion

Introduction

A review of the literature available up until 1986 on haemopoiesis and the growth factors believed to regulate this process is presented in Chapter 1. To bring this review up to date, more recent information on the biological activities and the molecular biology of the haemopoietic growth factors is discussed in this section.

The availability of quantities of the purified recombinant gibbon and human IL-3 has facilitated the study of the biological activities of IL-3 in primates. To date, the biological activities of gibbon and human IL-3 appear to be largely similar to those of mouse IL-3. Thus, human and gibbon IL-3 support the formation of G, GM, M, mixed and erythroid colonies in semi-solid culture (Kobayashi et al.,1988; Lopez et al.,1987). In humans, as in mice, there is a broad overlap in the activities of IL-3 and GM-CSF (Kobayashi et al.,1988; Migliaccio et al.,1988) However, at saturating levels of each factor, human IL-3 is superior to GM-CSF in promoting megakaryocyte-containing colonies (Kobayashi et al.,1988). Further differences between the two growth factors is evident in serum-free cultures in which IL-3 is 2-3 fold more active than GM-CSF in promoting erythroid burst growth in the presence of Epo, whereas GM-CSF is 2-3 fold more active than IL-3 in inducing GM colonies (Migliaccio et al.,1988).

In parallel with the mouse system, human Epo and G-CSF appear to be considerably more lineage-restricted than IL-3. Human Epo primarily stimulates the proliferation and terminal maturation of

CFU-E (Sonada et al.,1988). Human G-CSF supports the proliferation of colonies consisting primarily of neutrophils (Souza et al., 1986).

The biology of M-CSF has become something of an enigma over recent years. The synthesis of both human and mouse M-CSF is complex. In both species the gene for M-CSF transcribes multiple RNA species that range in size from approximately 1.5 to 4.4 kb (Kawasaki et al.,1985; Wong et al.,1987; Rajavashisth et al.,1987) As yet only one cDNA encoding mouse M-CSF has been isolated (Rajavashisth et al.,1987) but there are now three different human cDNAs described that encode biologically active M-CSF (Kawasaki et al.,1985; Wong et al.,1987; Ceretti et al.,1988). The human M-CSF cDNAs, referred to as M-CSFα, M-CSFβ and M-CSFγ, are probably the result of alternative RNA splicing within the coding region of the gene and encode proteins of 256 (Kawasaki et al.,1985), 554 (Wong et al.,1987) and 438 (Ceretti et al.,1988) amino acids. The two predominant proteins synthesised from these cDNAs are 44 kd (M-CSFβ, M-CSFγ) and 28 kd (M-CSFα). Each of the M-CSF molecules is represented by a form anchored to the plasma membrane and soluble M-CSF is processed from the membrane bound precursor (Rettenmier et al.,1987; Ceretti et al.,1988).

The functional significance of these different forms of M-CSF remains to be determined although Ceretti et al. (1988) have speculated that the three membrane bound precursors may present different proteolytic cleavage sites that are cleaved by different proteases located at different sites in the body. On the other hand, one or more of the membrane bound forms may be functionally active requiring direct cell-to-cell contact for binding to M-CSF

receptors on adjacent cells to initiate a biological response. The distance between cells required for this interaction may be critical and would vary according to the form of M-CSF expressed.

Mouse M-CSF stimulates colonies consisting primarily of macrophages. However, at low concentrations, M-CSF also stimulates granulocyte and granulocyte/macrophage colonies (Chapter 4). Furthermore, in the presence of IL-1, M-CSF generates CFC for the megakaryocyte and granulocyte lineages in addition to M-CFC (McNiece et al.,1987). Therefore, the activity of M-CSF is not restricted to cells committed to the macrophage lineage. Recombinant human M-CSFs also stimulate macrophage colony growth although paradoxically the human factors have much less biological activity on human bone marrow cells than on comparable populations of mouse bone marrow cells (Wong et al.,1987; Ceretti et al.,1988). In view of the synergism of mouse M-CSF with IL-1 and IL-3 (reviewed in Chapter 1 and below), it is possible that human M-CSF requires the presence of another factor(s) for optimal stimulation of human target cells.

As reviewed in Chapter 1, recombinant mouse IL-4 and IL-5 support the proliferation of mast cells and eosinophils respectively. Human IL-5 has been shown to have identical biological activities to those of the mouse factor (Campbell et al.,1987) but the activity of IL-4 on human mast cells has yet to be demonstrated. Recently, IL-6 was added to the list of myeloid growth factors. Human IL-6, first identified in the basis of its antiviral activity, supports the proliferation of some but not all mouse granulocyte/macrophage progenitors although it has little proliferative activity on human

target cells (Wong et al.,1988). As yet no data is available on the activity of mouse IL-6 on myeloid cells. The interactions of the haemopoietic growth factors with the various cell lineages is summarised in Fig 8.1.

In situ hybridisation studies have localised the human genes for IL-3 (Le Beau et al.,1987), GM-CSF (Huebner et al.,1985), IL-5 (Sutherland et al.,1988), IL-4 (van Leeuwen et al.,1989), M-CSF Pettenati et al.,1987), and the proto-oncogene *fms* (Le Beau et al.,1986), which is believed to be the M-CSF receptor, to the segment of chromosone 5 between bands 5q23 and 5q33. The IL-3 and GM-CSF genes are only 9 kb apart (Yang et al.,1988) and the IL-4 and IL-5 genes are 90 - 240 kb apart (van Leeuwen et al.,1989). The close physical linkage between these genes is also found in the mouse where IL-3 and GM-CSF are only 14 kb apart (Lee and Young, 1989) and IL-4 and IL-5 are 110 - 180 kb apart (Lee et al.,1989). The proximity of the four related genes suggests that these genes may have evolved by ancient gene duplication. Furthermore, since the relatively close IL-3/GM-CSF and IL-4/IL-5 linkages are conserved in mouse and human, these relationships may be important for the regulation of expression of these genes.

The remainder of this Chapter reviews the proliferative and synergistic activities of IL-3 in the light of recent developments including those presented in this thesis. This is followed by a consideration of the possible physiological role and therapeutic potential of this factor. The final section outlines suggestions for further work using the A/J model.

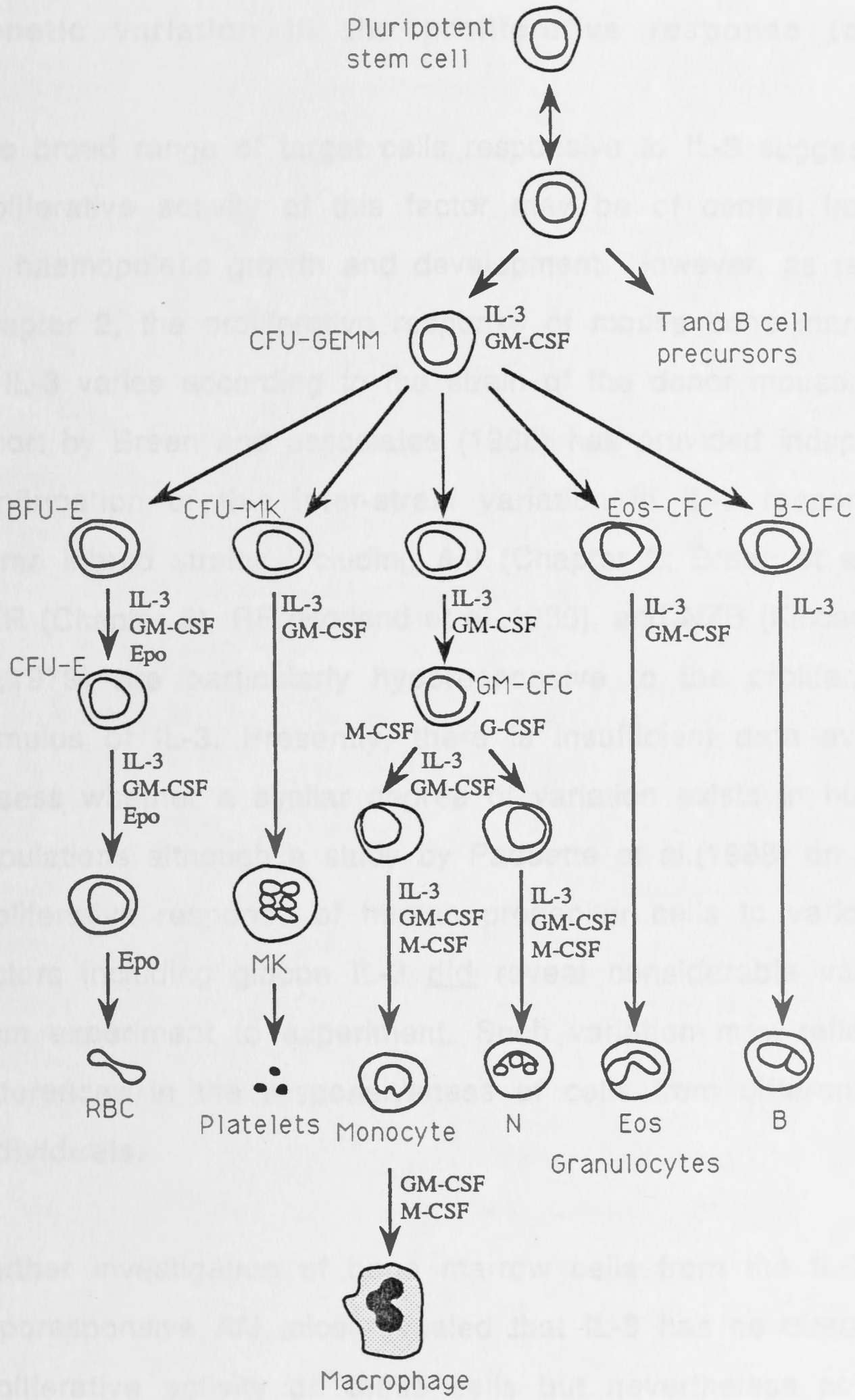


Fig 8.1 Scheme of haemopoiesis (adapted from Clark and Kamen, 1987). The sites of action of the different growth factors are deduced from analysis of mature cells found in colonies grown in the presence of the factors.In the presence of other factors M-CSF and G-CSF will also act on relatively early progenitor cells (see text). RBC, red blood cell; N, neutrophil; Eos, eosinophil; B, basophil; MK, megakaryocyte.

Genetic variation in the proliferative response to IL-3

The broad range of target cells responsive to IL-3 suggests that the proliferative activity of this factor may be of central importance for haemopoietic growth and development. However, as revealed in Chapter 2, the proliferative response of mouse bone marrow cells to IL-3 varies according to the strain of the donor mouse. A recent report by Breen and associates (1988) has provided independent confirmation of this inter-strain variation in IL-3 responsiveness. Some inbred strains including A/J (Chapter 2; Breen et al.,1988), AKR (Chapter 2), RF (Horland et al.,1980), and NZB (Kincade et al.,1979) are particularly hyporesponsive to the proliferative stimulus of IL-3. Presently, there is insufficient data available to assess whether a similar degree of variation exists in human populations although a study by Paquette et al.(1988) on the proliferative response of human progenitor cells to various growth factors including gibbon IL-3 did reveal considerable variation from experiment to experiment. Such variation may reflect differences in the responsiveness of cells from different individuals.

Further investigation of bone marrow cells from the IL-3-hyporesponsive A/J mice revealed that IL-3 has no direct proliferative activity on these cells but nevertheless acts synergistically with M-CSF, maintains the viability of growth factor responsive cells and induces growth factor expression in A/J bone marrow cell cultures. Since IL-3 does not induce the proliferation of bone marrow cells from some strains, the direct mitogenic activity of IL-3 is probably not essential for maintaining

normal haemopoiesis. Nevertheless, the synergistic, maintenance, and growth factor-inducing activities of IL-3 may play an important role in controlling blood cell growth and development.

Synergistic activities of IL-3

Mouse IL-3 acts synergistically with M-CSF to stimulate the production of distinctively large colonies from a unique population of haemopoietic progenitors termed high proliferative potential colony forming cells (HPP-CFC). Properties of HPP-CFC, including their relative resistance to 5-FU *in vivo*, suggest that they comprise a developmentally early cell population in the haemopoietic system (Bradley and Hodgson,1979; McNiece et al.,1986). In addition, the predominant growth factor responsive CFC found in the yolk sac blood islets of 8 day mouse embryos, responds synergistically to the combination of IL-3 plus M-CSF (Townsend et al., unpublished observations). These data emphasise that the activities of M-CSF are not restricted to late progenitor cells. As yet it is not known whether the interaction between IL-3 and M-CSF also occurs in the human system. Both human and mouse IL-3 also act synergistically with erythropoietin to enhance erythroid colony formation (Sonada et al.,1988; Iscove et al.,1982).

To explain these synergistic interactions, Iscove proposed that IL-3 acts as a multilineage factor which supports the proliferation and maturation of early haemopoietic precursors, whereas lineage restricted factors such as M-CSF or erythropoietin act only on cells

which have acquired the appropriate receptors as part of their differentiation programme. As a corollary, the synergistic activity of IL-3 is dependent on its proliferative activity on an early haemopoietic precursor population.

However, bone marrow cells from the IL-3-hyporesponsive strains produce distinctively large colonies in response to IL-3 plus M-CSF, indicating that the proliferative activity of IL-3 is not an essential requirement for this synergistic interaction (Chapter 4). The results of experiments reported in Chapter 5 suggest that IL-3 synergises with M-CSF primarily by enhancing M-CSF receptor expression although other mechanisms may also be involved.

The synergistic interaction between IL-3 and M-CSF also occurs *in vivo*. Thus, although relatively large concentrations of IL-3 or M-CSF must be administered to produce a detectable effect *in vivo*, these concentrations can be vastly reduced by administering the factors together (Broxmeyer et al.,1987). The effects of the administration of low concentrations of IL-3 plus M-CSF include an increase in the proliferation of HPP-CFC *in vivo* (Williams et al.,1987b).

In agreement with Iscove's model, the synergism between IL-3 and Epo is apparently correlated to the proliferative activity of IL-3 (Chapter 6). Since IL-3 and Epo do not enhance erythroid colony formation from A/J bone marrow cells, alternative pathways must exist for the regulation of erythropoiesis (discussed in Chapter 6).

The synergistic activities of IL-3 extend beyond its interactions with M-CSF and erythropoietin. Mouse IL-3 and human G-CSF act synergistically to support the proliferation of mouse multipotential stem cells (Ikebuchi et al.,1988) indicating that the action of G-CSF is not restricted to commited precursors of the granulocyte lineage. The combination of gibbon IL-3 with human G-CSF or GM-CSF results in synergistic stimulation of colony formation from human myeloid precursor cells (Paquette et al.,1988). In humans and mice, the combination of IL-6 and IL-3 accelerates the appearance of blast cell colonies in culture (reviewed in Wong and Clark,1988). IL-3, GM-CSF and M-CSF all act synergistically with a growth factor found in the medium conditioned by a mouse adherant cell line, TC-1 (Quesenberry et al.,1987). The mechanisms involved in these synergistic activities of IL-3 are as yet unknown.

IL-1 (formerly known as haemopoietin-1) and IL-4 belong to a separate class of haemopoietic factors which have no intrinsic colony stimulating activity but enhance the response of haemopoietic cells to various growth factors including IL-3 (reviewed in Chapters 1 and 6). The relevance of the synergistic interaction between IL-3 and IL-4 is questionable however, since IL-4 does not enhance IL-3-responsiveness in the A/J system.

Role of IL-3 in haemopoiesis

Constitutive haemopoiesis

As discussed in Chapter 1, as a prerequisite to ascribing a physiological role for IL-3 in the development and maintenance of the haemopoietic system, it must be demonstrated that cells present at the major sites of constitutive haemopoiesis are capable of producing this factor. Recent work indicates that endothelial cells which are present in the bone marrow stroma and surround blood islets in the yolk sac may, under certain conditions, be responsible for IL-3 production. A CSF with the chromatographic and biological properties of IL-3 has been isolated from the conditioned media produced by cultures of human umbilical vein endothelial cells (Elsbury et al., unpublished observations). This is consistent with a report by Labastie et al (1984) that an IL-3-like activity can be detected in yolk sac (reviewed in Chapter 1). In addition, B cells have been identified as a source of spontaneously exfoliated, membrane associated growth factors including an erythroid burst stimulatory activity which may be identical to IL-3 (Dainiak et al.,1987). As numerous B cells reside in the bone marrow they too are a potential source of IL-3 for constitutive haemopoiesis. However, these data have yet to be confirmed in other laboratories

Does IL-3 regulate the self-renewal and differentiation of haemopoietic stem cells? A number of investigators have shown that the targets of IL-3 include stem cell populations. For example, mouse IL-3 supports the growth of blast cell colonies *in vitro* and

stimulates the proliferation of CFU-S both *in vitro* and *in vivo* (reviewed in Chapter 1). Similarly human IL-3 and gibbon IL-3 support the proliferation of multipotential stem cells with extensive replating capacity *in vitro* (Kobayashi et al.,1988; reviewed in Clark and Kamen,1987). However, since IL-3 has no direct proliferative activity on haemopoietic cells from a number of mouse strains (Chapters 2 and 3), it is unlikely that this activity plays an essential role in stem cell regulation. This is consistent with a recent report by Zipori and Lee (1988) aimed at determining the influence of IL-3 gene expression on the ability of a stromal cell line, 14F1.1, to sustain haemopoiesis. 14F1.1 cells are a line of endothelial-adipocytes which produce M-CSF but not IL-3, GM-CSF, or G-CSF. This stromal line is capable of maintaining long term haemopoiesis when co-cultured with bone marrow cells. Zipori and Lee found that IL-3 gene expression, achieved by transfection of 14F1.1 cells with a plasmid carrying an IL-3 cDNA, did not modify the ability of these cells to maintain stem cell renewal.

Evidence to date suggests therefore, that IL-3 is not an important regulator of stem cell renewal. Other more plausible candidates for this role include the endogenous CFU-S cell cycle stimulating and inhibitory factors (reviewed in Chapter 1) and as yet uncharacterised factor(s) produced by stromal cell lines, such as 14F1.1, which are capable of maintaining long term haemopoiesis.

On the other hand there <u>is</u> evidence that IL-3 regulates the <u>differentiation</u> of stem cells. For example, in the study by Zipori and Lee (1988), the expression of IL-3 in the stromal cell/bone marrow cell co-cultures resulted in a shift in differentiation

towards the myeloid lineages. This effect may be accounted for partly by an enhancement of the response of bone marrow cells to the endogenous M-CSF present in these cultures. As mentioned earlier, IL-3 also appears to enhance the M-CSF-responsiveness of stem cells found in the yolk sac blood islets of 8 day mouse embryos, suggesting that this differentiative activity is involved in early haemopoietic development. Perhaps then, the major role of IL-3 in constitutive haemopoiesis is to facilitate the differentiation of stem cells towards myeloid lineages. In the bone marrow microenvironment the final outcome of this effect would depend on which other endogenous factors were present.

Inducible haemopoiesis

As a product of activated T cells, IL-3 is probably important for host defense rections against the invasion of foreign substances. However, the role of IL-3 in this process is not clear since GM-CSF, which shares many of the activities of IL-3, is produced by the same population of activated T cells.

One of the activities of IL-3 which is apparently unique to this factor is its proliferative and differentiative activity on mucosal mast cells *in vitro*. Exogenous administration of IL-3 also has a striking effect on mast cell numbers *in vivo* (Metcalf et al.,1986a). Therefore, IL-3 production may regulate the mast cell hyperplasia involved in parasite and allergic responses. The action of IL-3 on IL-4 expression by the mast cell-like 32D cl-23 cells (Chapter 7) adds a further dimension to the possible role of IL-3 in such responses. IL-4 is capable of increasing Ig E production by

activated B cells *in vitro* (Coffman et al.,1986) and probably *in vivo* (Finkelman et al.,1986). In turn, binding of Ig E to the high affinity receptor expressed on the mast cell mediates the release of vasoactive amines. Thus, if IL-3 induces IL-4 expression by mucosal mast cells *in vivo* this could represent an important mechanism for the amplification of Ig E mediated responses. The synergism between IL-3 and IL-4 on the proliferative response of mast cells could further enhance these responses.

The synergistic activities of IL-3 may also play an important role in inducible haemopoiesis by amplifying the inflammatory reponse induced by other factors. Such an effect would be particularly potent on the macrophage lineage since IL-3 not only enhances the proliferative response to M-CSF (Chapter 4) but also induces M-CSF expression by mature macrophages (Chapter 7).

Therapeutic potential of IL-3

Clinical trials are in progress to investigate the potential of growth factors, including IL-3, for enhancing the rate of bone marrow engraftment and for stimulating hematopoiesis in patients undergoing radiotherapy or chemotherapy for cancer. Bearing in mind the numerous synergistic activities of IL-3, it is likely that administration of this factor in combination with others would produce the greatest benefit. In this regard, treatment of monkeys with both IL-3 and GM-CSF results in an increase in neutrophils as well as platelets which is not seen in animals treated with GM-CSF alone (reviewed in Klingemann and Eaves,1988).

The administration of IL-3 may also result in adverse reactions. Indeed, in one mouse study, treatment with IL-3 resulted in increased replication of the spleen-focus-forming virus, probably by first increasing the cycling rates of the target cells for this virus (reviewed in Broxmeyer and Williams,1987). Unpleasant complications could also be caused by the differentiative activity of IL-3 on mast cells and macrophages and long term treatment with IL-3 may lead to enhanced myeloid differentiation at the expense of the lymphoid pathway.

As a final note of caution, the large intra-species variation in the response of mouse bone marrow cells to IL-3 may be reflected in the human population and should therefore be taken into consideration during analyses of clinical trials involving this growth factor.

Future use of the A/J model

Dissection of the signalling pathways induced by IL-3

The isolation of the proliferative from the synergistic and growth factor-induction responses to IL-3 in A/J bone marrow cells suggests that these reponses may be controlled by separate signalling pathways, perhaps via functionally different receptors. In support of this notion, Palazynski and Ihle (1984) reported the identification of IL-3 receptors of two different molecular weights, 55,000 and 70,000. Further binding analyses, including

Scatchard analysis, are required to determine whether there is any significant difference in the numbers, molecular weights, or binding affinities of IL-3 receptors on A/J bone marrow cells compared with those on bone marrow cells which proliferate in response to IL-3.

There is some evidence for the existence of two separate signalling pathways induced by IL-3. One of these putative signalling mechanisms involves the activation of protein kinase C. The interaction of IL-3 with its receptor on FDC-P1 cells results in the translocation and activation of protein kinase C (Farrar et al.,1985) and in the phosphorylation of two major cellular substrates with molecular weights of 68,000 (pp68) and 20,000 (pp20) (Evans et al.,1988). Phosphoaminoacid analysis demonstrated that pp68 was phosphorylated on threonine and not tyrosine residues. This eliminates the possibility that the phosphorylation observed had been affected by a tyrosine kinase and further implicates protein kinase C (a serine-threonine kinase) in the transduction of the signal induced by IL-3.

The best defined mechanism of activation of protein kinase C is the receptor-mediated hydrolysis of membrane bound phosphatidyl inositol 4,5-bisphosphate to generate two second messenger molecules: inositol triphosphate and diacylglycerol (Berridge and Irvine,1984). The release of inositol triphosphate causes a transient increment in the intracellular Ca^{2+} concentration, whereas the release of diacylglycerol activates protein kinase C. These two events act synergistically to elicit physiological responses. The combination of a tumor promoting phorbol ester and

a Ca^{2+} ionophore can mimic the effects of phosphoinositide breakdown: Phorbol esters directly activate protein kinase C (reviewed in Nishizuka,1984) and Ca^{2+} ionophores increase cytosolic Ca^{2+} levels.

Whetton et al. (1986) demonstrated that the IL-3-dependent cell line, FDCP-Mix 1, cultured with a phorbol ester, tetradecanoyl phorbol 13-acetate (TPA), and a Ca^{2+} ionophore (A23187) remained viable in the absence of IL-3 for 24 hr. At optimal concentrations TPA and A23187 also induced a small proliferative response in these cells although this was less than half the proliferative reponse induced by IL-3. From the observation that agents which mimic diacylglycerol and inositol triphosphate can partly replace IL-3 as a proliferation/survival signal, Whetton et al. inferred that IL-3 promotes the survival and proliferation of cells via its ability to stimulate phosphatidyl inositol metabolism. However, in recent work, Whetton et al. (1988) demonstrated that IL-3 does not stimulate an increase in inositol lipid turnover in FDCP-Mix 1 cells. Therefore, although activation of protein kinase C and a transient increase in cytosolic Ca^{2+} may be induced by IL-3, it is unlikely that these second messengers are generated via the phosphatidyl inositol pathway.

One possible explanation for the failure of IL-3 to induce proliferation of A/J bone marrow cells is that these cells are deficient in the generation or response to activated protein kinase C and/or increases in cytosolic Ca^{2+}. Some preliminary experiments were carried out during the course of this thesis to ascertain the effect of the phorbol ester, phorbol 12-myristate 13-

acetate (PMA), and the Ca^{2+} ionophore, A23187, on the proliferation of A/J and BALB/c bone marrow cells. Because the proliferation induced by these agents, alone or in combination, was somewhat variable and only of very low level in both A/J and BALB/c bone marrow cell cultures, the results are not formally presented elsewhere in this thesis. However, the general trends observed in these experiments are summarised below. Even at optimal concentrations, PMA (2ng/ml) alone had little effect on the proliferation of A/J or BALB/c bone marrow cells. PMA (2ng/ml) and ionophore (700ng/ml) acted synergistically to induce a low level of bone marrow cell proliferation. The response to this combination of agents was similar in both A/J and BALB/c bone marrow cell cultures suggesting that A/J cells respond normally to activated potein kinase C and cytosolic calcium fluctuations. Concentrations of calcium ionophore in the range 1-1,000 ng/ml failed to induce proliferation. At the higher concentrations in this range, ionophore actually inhibited background proliferation (proliferation occurring in the absence of exogenous growth factor) and inhibited the proliferation induced by IL-3 in both A/J and BALB/c cultures. Interestingly, PMA appeared to enhance the proliferation induced by IL-3 in A/J bone marrow cell cultures such that optimal concentrations of both PMA (2ng/ml) and IL-3 (100U/ml) induced a similar level of proliferation to that obtained with optimal concentrations of PMA and ionophore. In contrast, PMA only enhanced the IL-3-induced proliferation of BALB/c bone marrow cells when low concentrations of IL-3 were used (<5U/ml). This may indicate that A/J cells fail to generate normal levels of activated protein kinase C in response to IL-3. To investigate whether it is likely that the deficiency in A/J bone marrow cells

lies in the activation of protein kinase C, the threonine phosphorylation of cellular substrates induced in A/J cells by IL-3 should be compared with those induced in BALB/c cells. This may reveal whether the activation of protein kinase C is a critical event in the proliferative response to IL-3. The similarity in the response of A/J cells to PMA plus IL-3 and PMA plus ionophore, may indicate that IL-3 causes a transient increase in calcium levels (as does ionophore). Alternatively, the apparent interaction between IL-3 and PMA may be indirect because IL-3 induces the endogenous production of M-CSF in A/J bone marrow cell cultures (Chapter 7) and phorbol esters are known to enhance the proliferation induced by low concentrations of M-CSF (Stuart et al.,1981).

The other pathway implicated as the transducing mechanism for the proliferative signal of IL-3 involves a tyrosine kinase. IL-3 specifically induces tyrosine phosphorylation of a 150,000 kd membrane glycoprotein (gpp150) in the IL-3 dependent cell lines IC2 and DA-1 (Koyasu et al.,1987) This tyrosine phosphorylation occurs earlier than the threonine phosphorylation of pp68 and pp20. Again, a comparison of the cellular substrates phosphorylated after treatment of A/J and BALB/c bone marrow cells with IL-3 may reveal whether the tyrosine kinase pathway is critical for IL-3-induced proliferation.

Further analysis of the interaction between IL-3 and other factors

The separation of the mitogenic activity of IL-3 from some of its synergistic activities in A/J bone marrow cell cultures should facilitate further analysis of the interactions between IL-3 and

other growth factors. In particular, the A/J model should facilitate the isolation and characterision of the HPP-CFC responsive to IL-3 and M-CSF.

Analysis of the importance of IL-3-induced proliferation in host defense reactions

A/J mice should provide a model for studying the importance of the proliferative response to IL-3 in host defense reactions. Previous reports have shown that A/J mice are highly susceptible to a variety of intracellular parasites such as Schistosoma mansoni (James et al.,1983), Rikettsia akari (Meltzer et al.,1982), and Legionella pneumophila (Yamamoto et al.,1988). Much of this impaired resistance may be accounted for by the observation that IFN-γ does not efficiently induce cytotoxic activity in macrophages from these mice (Hamilton et al.,1986). However, Meltzer and colleagues (1982) demonstrated that the defective cytotoxic activity displayed by macrophages from A/J mice is not causally related to the extreme susceptibility of these mice to Rikettsia akari. Further study is required to assess the involvement of the direct mitogenic activity of IL-3 in resistance to these bacterial infections. In view of the apparent dependency of mucosal mast cells on IL-3 for proliferation it would also be particularly interesting to study the susceptiblity of A/J mice to helminth parasite infection.

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